The social life of ABC transporters

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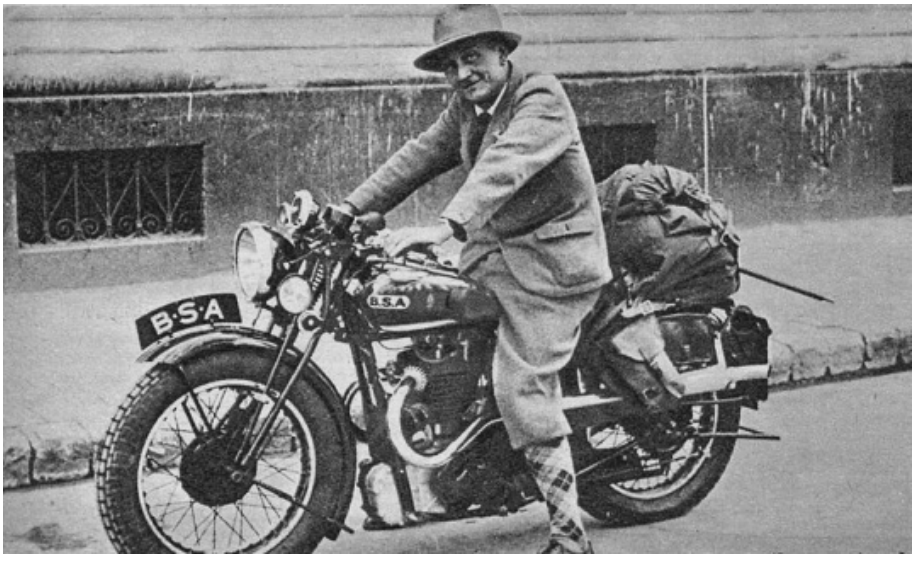

Content

Abbreviations

20:4 LPE	1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine
20:4 PE	1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine
22:6 LPE	1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine
22:6 PE	1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine
3T3	Mouse embryonic fibroblast cells
ABC	ATP Binding Cassette
ADMET	Absorption, distribution, metabolism, excretion, toxicity
AM	Acetoxymethyl ester
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
BCRP	Breast cancer resistant protein or ABCG2 or MXR
BHK	Baby Hamster Kidney fibroblast cells
BSA	Bovine serum albumin
BSEP	ABCB11 transporter
Cav-1	Caveolin-1
CD	Methyl-β-cyclodextrin
CDC	Methyl-β-cyclodextrin filled with cholesterol
CEM	Cholesterol enriched membranes
CFDA	5-carboxyfluorescein diacetate
CFTR	Cystic fibrosis transmembrane conductance regulator or ABCC7
Cho	Cell line initiated from a biopsy of an ovary of an adult Chinese hamster
CNS	Central nervous system
CO	Cholesterol oxidase
CSA	Cyclosporin A
DDI	Drug-drug interaction
DIG	Detergent-insoluble glycosphingolipids
DNA	Dezoxiribonucleic acid
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
DRM	Detergent resistant membranes
EGTA	Glycol ether diamine tetraacetic acid
EM	Electron microscopy
EMA	European Medicines Agency
ERM	Ezrin, radixin and moesin
FCS	Fetal calf serum
FDA	U.S. Food and Drug Administration
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GPI	Glycosylphosphatidyl inositol
GSH	Glutathione
HBSS	Hank’s balanced salt solution
HEK293	Cell line originally derived from human embryonic kidney cells
HeLa	Cervical cancer cells derived from Henrietta Lacks in 1951

HIS-tag	String of six to nine histidine residues
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
IO	Inside-out vesicles
ISP-1	Myriocin
ITC	International Transporter Consortium
K_m	Michaelis constant, the substrate concentration at which the reaction rate is half of V_{max}
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization tandem mass spectrometry
LO	Lovastatin
LPE	Lysophosphatidylethanolamine
LTC_4	Cysteinyl leukotriene C_4
MAF	Multidrug activity factor
MD	Microdialysis
MDCKII	Madin Darby canine kidney epithelial cells derived from dog
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1 or Pgp or ABCB1
MOPS	4-morpholinepropanesulfonic acid
MRP1	Multidrug resistance-associated protein 1 or ABCC1
MTT	Thiazolyl blue tetrazolium bromide
M-β-CD	Methyl-β-cyclodextrin
NBD	Nucleotide binding domain
NIH	National Institutes of Health
NIH	National Institutes of Health
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDMP	D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
PFA	Paraformaldehyde
PG	Phosphatidylglycerol
Pgp	P-glycoprotein or MDR1 or ABCB1
PI	Phosphatidylinositol
PIP2	Phosphatidylinositol 4,5 bisphosphate
PLAP	Placental alkaline phosphatase
PS	Phosphatidylserine
R123	Rhodamine 123
R_{con}	Fluorescence intensity slope of accumulating calcein
Rho-GDI	Rho GDP-dissociation inhibitor protein
R_{inh}	Fluorescence intensity slope of accumulating calcein when the transporter is inhibited
RO	Right-side-out vesicles

RO	RO 48-8071
SDS	Sodium dodecyl sulfate
Sf9	Insect cell line derived from *Spodoptera frugiperda*
siRNA	Small interfering RNA
SPT	Single particle tracking
TCA	Trichloroacetic acid
TCR	T-cell receptor
TIFF	Triton-insoluble floating fraction
TIM	Triton-insoluble membranes
Tm	Melting temperature
TMD	Transmembrane domain
V_{max}	The maximum rate or speed achieved by the system at maximum (saturating) substrate concentration
VP16	Etoposide

Chapter 1

About ABC transporters: Some reflections and scope of the thesis

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Part of this Chapter has been submitted

Chapter 1

Abstract

ATP binding cassette (ABC) transporters are transmembrane proteins that are ubiquitously present in both prokaryotic and eukaryotic cells. Within eukaryotic cells, they translocate a variety of natural substrates, including lipid species, across plasma and Golgi membranes. However, they are particularly known for their ability to convey multidrug resistance when tumors are treated with cytostatic drugs, thus frustrating therapeutic treatment of this disease. As a result, extensive investigations have been carried out, aimed at understanding the structure/function properties of the transporters in order to develop strategies to annihilate their activity at such conditions. Indeed, as one of the first known membrane transporters, the protein structure of some ABC transporters (e.g. Pgp) has been elucidated in detail. Consequently, in depth and novel molecular mechanisms have been clarified underlying the transporter's ability to process different kinds of substrates. Here, we present a brief overview, highlighting the different approaches in research that have made ABC transporters popular and rewarding for detailed investigations in recent years. The chapter is concluded with an account of the scope of this thesis.

I. Why does one study ABC transporters?

I.A. Cancer, role of ABC transporters, overview of transporters

ABC transporters form one of the largest protein families. 49 ABC coding genes have been identified in the human genome, but they are present in all known organisms from bacteria to humans (1). The notion that they are so widely present and that they have relatively conserved functions suggests that they are of utmost importance.

Most proteins of this family consist of two domains (Fig. 1): the so called transmembrane domain (TMD), which contains 6 transmembrane helixes, and the nucleotide (ATP) binding domain (NBD). Furthermore, whole-transporters (12 helixes and 2 ATP binding domains) and half-transporters (6 helixes and 1 ATP binding domains) are distinguished. The latter must form either homodimers (even trimers or tetramers) or heterodimers to form a functional transporter.

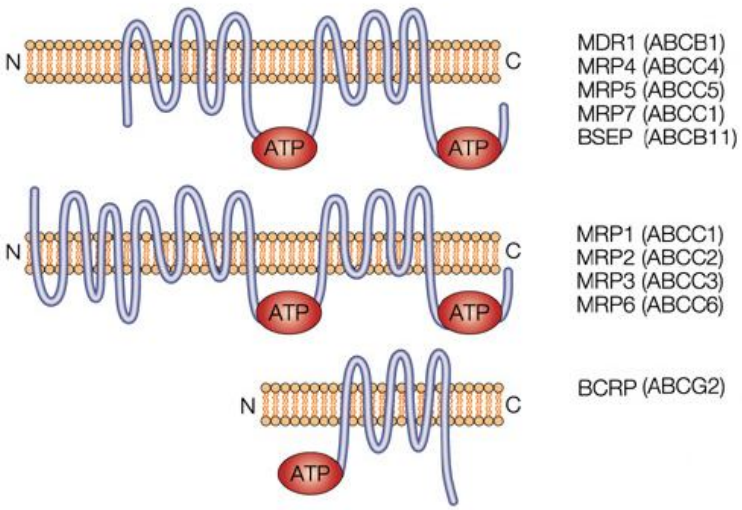

Figure 1. Basic representation of ABC transporters structure
(adopted from Gottesman et. al. 2002)

ABC transporters are primary active transporters, i.e., they utilize energy from Adenosine-5'-triphosphate (ATP) hydrolysis to pump molecules through the membrane against a concentration gradient (2). Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds over membranes either to the cell interior, as part of a metabolic process, or from inside to outside the cell, in case of transport to other organs or secretion from the body.

Over the last 15 – 20 years, extensive research in this area resulted in a significant advance in our understanding of how different ABC transporters modulate the different pharmacokinetic parameters of drugs (3). ABC transporters are widely studied in the context of multidrug resistance (MDR) of tumors: cancer cells are rescued from chemotherapy by overexpression of one or more transporters that pump chemotherapeutic drugs out of the cell. In fact, the nature of cancer cells helped us to understand why these transporters are so widely distributed, since they provide protection against xenobiotics.

By aligning the amino acid sequences of the NBD domains and performing phylogenetic analysis, the eukaryotic ABC transporter genes can be grouped into seven major subfamilies, named A-F. Because the three most important transporters responsible for MDR are Pgp, MRP1 and Breast cancer resistant protein (BCRP), and while the research presented in this thesis mainly focuses on MRP1 and Pgp, we will first highlight the latter two proteins in some further detail.

P-glycoprotein (Pgp)

Depending on nomenclature, Pgp is also known as MDR1 (see Fig. 1, top structure) or ABCB1, and was the first human ABC transporter cloned (in 1976) and characterized through its ability to confer an MDR phenotype to cancer cells. The glycosylated transporter is located in crucial body-environment interfaces, like the luminal membrane of the small intestine and the blood-brain barrier, and in the apical membranes of excretory cells such as hepatocytes and kidney proximal tubule epithelia (4). Pgp has an important role in limiting entry of various drugs into the central nervous system. In addition, the protein is also closely involved in intestinal absorption and in biliary and urinary excretion of drugs. It recognizes hydrophobic substrates, including drugs such as colchicine,

etoposide (VP16), adriamycin, and vinblastine as well as lipids, steroids, xenobiotics, and peptides.

Multidrug resistance-associated protein (MRP1)

MRP1 or ABCC1 is also known for its prominent role in multidrug resistance. This full transporter has an additional 6 transmembrane helices (see Fig. 1, middle structure) and is the principal transporter of glutathione-linked compounds out of cells. Other substrates are glucuronides and sulfate conjugates. The MRP1 pump confers resistance to among others doxorubicin, daunorubicin, vincristine, and colchicines, very similar to the profile of Pgp, except that in this case the transported drugs are conjugated to glutathione. MRP1 can also transport leukotrienes such as cysteinyl leukotriene C_4 (LTC_4), and is therefore thought, apart from protecting cells from chemical toxicity and oxidative stress, to mediate inflammatory responses involving cysteinyl leukotrienes.

I.B. ADMET properties, commercial activity and clinical significance

The biochemical and clinical investigations of ABC transporters are of top priority, since chemotherapy is still one of the most important treatments against cancer. Furthermore ABC transporters located at different important pharmacological barriers (Fig. 2) can influence the Absorption–Distribution–Metabolism–Excretion-Toxicity (ADMET) properties of drug molecules.

These facts draw the attention of pharma and biotech companies to extensive research in the structure and function of ABC transporters. For example, such work has led to the discovery that drug-transporter interactions can predict the tissue distribution of drug candidates. Thus ABC transporters in the canalicular membrane of hepatocytes mediate the hepatic efflux of drugs, bile salts and metabolites, while cytokines affect the expression levels of these proteins (5). Hence ABC transporters are key determinants of drug metabolism and drug clearance by the liver.

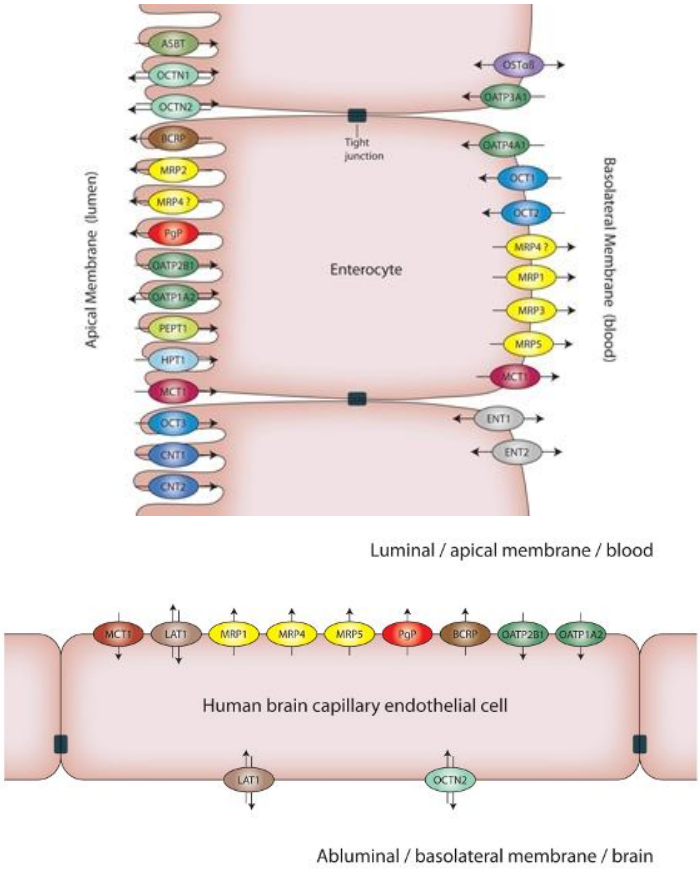

Figure 2. Schematic models of pharmacological barriers
Transporters in the human intestinal barrier (upper) and in the blood-brain-barrier (lower). (Solvo.com)

This knowledge and insight is of utmost importance for industry because of the 'fail fast – fail cheap' paradigm, and the need for high-throughput methods, which are able to measure drug-drug and drug-transporter interactions, recognizing substrates or inhibitors.

For this purpose, a wide range of transporters in different expression systems, cell lines and animal models have been constructed for subsequent analysis by biotech companies like Solvo Biotechnology, BD Biosciences or Genomembrane.

The transporter related market is growing (6), because there is an emerging need for these products and services in drug development industry. This is further fuelled by the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA) and

the International Transporter Consortium (ITC). These institutions formulated guidelines and critical recommendations that industry should follow when developing new drugs.

ITC scientists focused on transporters that have demonstrated a role in pharmacokinetics and drug-drug interactions (DDIs) in published studies. ABC transporters of emerging clinical importance in the absorption and disposition of drugs are: Pgp (ABCB1), BCRP (ABCG2), BSEP (ABCB11), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), and MDR3 (ABCB4). They claim that "The identification of membrane transporters that influence the disposition and safety of drugs is a new challenge for drug development programs, as well as for regulatory agencies worldwide. Drug transporter information is becoming common in drug labels, and provides important mechanistic ADME information that is useful for patients, physicians, regulatory agencies and research scientists." (4).

According to the FDA, Pgp, organic anion transporter (OAT), organic anion transporting polypeptide (OATP), organic cation transporter (OCT), MRP-s, and BCRP are the most relevant transporters (7). According to this agency drug-transporter studies in just one system are not appropriate, but a sequence of studies should be planned, ranging from *in vitro* studies to *in vivo* human studies. The FDA agrees that negative results from early *in vitro* and early clinical studies can eliminate the need for later clinical investigations, a cost effective measure for the pharma industry. They pay much attention to Pgp that affects the pharmacokinetics and pharmacodynamics of the co-administered drugs. For example by modulating Pgp, the oral bioavailability and brain uptake of drugs can be influenced.

An important aim in ABC transporter research is to find inhibitors and apply them together with chemoterapeutic agents. By doing so, the transporter related efflux could be decreased and hence, the effectiveness of treatment increased. The first generation of inhibitory compounds, such as R-verapamil or the immunosupressant Cyclosporin A were already used in the clinic for other purposes, and were only later shown to be an inhibitor of Pgp or MRP1 (for example). Unfortunately, because of the high concentrations needed to obtain effective inhibition, the application of these inhibitors was not feasible because of side effects. The second generation of inhibitors, including R-verapamil and the cyclosporin analog PSC833, could be used at lower concentrations. The third generation of

drugs is based on developments involving novel hydrophobic peptide chemosensitizers (reversins 121 and 205) structures (8). However, as yet, it is unclear whether the clinical effective dose of these modulators is tolerable. Theoretically, monoclonal antibodies against ABC transporters might be effective in the battle against MDR. Primarily those antibodies could be useful, which bind to extracellular epitopes of the specific ABC transporters and inhibit their function (9).

ABC transporters do not only cause problems when they function well. Mutations in ABC genes, causing malfunctioning of the transporter protein, are the origin of several diseases, summarized in Table 1. For example the ABCA1 transporter is thought to be involved in the shuttling of cholesterol and phospholipids from cells to high-density lipoprotein (HDL) particles. Different mutations in the ABCA1 gene cause Tangier disease. Patients with this syndrome are characterized by a deficient efflux of lipids from macrophages, resulting in an early onset of atherosclerosis and low levels of HDL in the blood (10). There are other inherited lipid-linked disorders, connected with ABC transporters. In the case of progressive familial intrahepatic cholestasis several transporters are affected: ABCB4 is required for the transport of phosphatidylcholine from the bile canalicular membrane of hepatocytes onto bile micelles. ABCB11 transfers bile acids, while cholesterol is exported by a heterodimer of ABCG5 and G8. Mutations in the genes coding for the latter two proteins can cause sitosterolemia. In this case the transport of sterols back into the intestinal lumen is disrupted (11).

Cystic fibrosis is caused by a mutation in the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR or ABCC7). This protein is required to regulate secretion components of sweat, digestive juices and mucus. The disease affects the lungs, pancreas, liver and intestine and is characterized by abnormal transport of chloride and sodium across epithelium, leading to thick, viscous secretions (12).

Name	Alias	Disease
ABCA1	ABC1	Tangier disease
ABCA4	ABCR	Retinitis pigmentosa, Stargardt disease
ABCB4	MRD3	Intrahepatic cholestasis
ABCB7	ABC7	Sideroblastic anemia with ataxia
ABCB11	BSEP	Intrahepaticus cholestasis
ABCC2	MRP2	Dubin-Johnson syndrome
ABCC6	MRP6	Pseudoxanthoma elasticum
ABCC7	CFTR	Cystic fibrosis
ABCD1	ALD	Adrenoleukodystrophy
ABCG5	Sterolin1	Sitosterolemia
ABCG8	Sterolin2	Sitosterolemia

Table 1. Short summary of ABC transporter malfunction related diseases

I.C. Role of lipid rafts, cholesterol and cytoskeleton

Although much insight has been gained in ABC transporter-triggered processes, there is still much to be learned about the molecular mechanism(s) of action. Investigating the proteins and their interactions with neighboring molecules can help to develop new and more effective inhibitors.

Research over the past decades has led to substantial adjustments of the fluid mosaic model of membranes, as originally proposed by Singer and Nicolson. The plasma membrane of cells is no longer thought to be an ocean of randomly organized lipids and proteins, seen as “icebergs” floating around. Rather it has been recognized that membranes are consisting of a patchwork of highly dynamic domains, and containing lipid enriched substructures that are known as lipid rafts. These rafts, which are thought to vary in size from 20 to 200 nm, appear to contain relatively high amounts of cholesterol, (glyco)sphingolipids and the more highly saturated phospholipids and distinct sets of proteins thus forming a tightly packed membrane domain. Probably the latter quality has led to their discovery: historically, rafts are defined as low density membrane fractions that are resistant to solubilization in ice cold 1 % Triton X-100, a nonionic detergent. Accordingly, in the literature there are usually a variety of definitions that refer to these domains, like Triton-insoluble membranes (TIM), Triton-insoluble floating fraction (TIFF),

detergent resistant membranes (DRM), detergent-insoluble glycosphingolipids (DIG) or cholesterol enriched membranes (CEM). Lipid rafts are interesting targets for research because they play a role in many cellular processes, including cellular internalization (e.g. via caveolae) and cell signaling. Lipid raft signaling is believed to be involved in diseases such as Parkinson's, Alzheimer's and Human immunodeficiency virus (HIV) disease. Extensive research has developed this field, the overall conclusion being that rafts can exert both positive and negative effects on signal transduction.

In spite of the importance of the lipid raft concept in cell biology, there is still a lot of discussion and controversy concerning the nature and even the existence of these membrane domains (13). There is no unifying model, probably because scientists work with fairly indirect and different approaches. Apart from Triton X-100, a wide variety of other detergents have been applied to isolate membrane raft fractions, including Lubrol, NP-40 and Brij 98. Strong criticisms against the lipid raft hypothesis were based on the notion that low temperature and detergents may induce lipid reorganization during the isolation of rafts, and therefore induce artifacts. Novel approaches for lipid raft isolation were developed, which obviated the need of detergents, the so called detergent-free raft isolation procedures. Given the dynamics and the great variety of isolation procedures, it is not at all surprising that an equal variety in the composition of rafts has been reported.

Despite these problems, there are indications that lipid rafts bear relevance for ABC transporter function. ABC transporters are partially localized in lipid rafts. This suggests that major lipid raft components, like cholesterol and/or (glyco)sphingolipids, are important for ABC transporter function. Alternatively, it is also possible that the more tightly packed and less "liquid-like" milieu is necessary to achieve the correct conformation of the transporter protein for expressing its activity.

Yet, given that lipid rafts are considered to be connected to the actin cytoskeleton beneath the plasma membrane other factors may also have to be taken into account. There are two structural organizations of actin in the cell. First the stress fibers, that span the whole cell providing it with a kind of skeleton for anchorage to the substrate. Secondly the cortical actin (Fig. 3), which is localized as a network just beneath the plasma membrane.

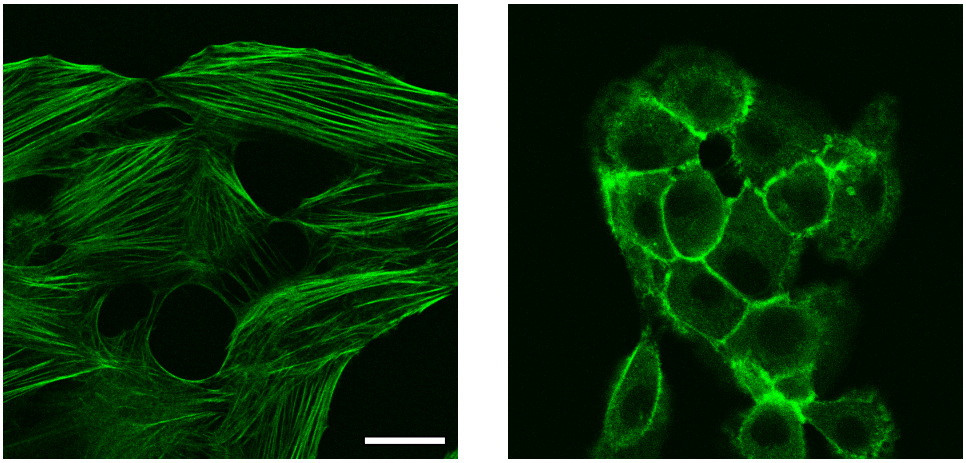

Figure 3. Confocal images showing actin stress fibers (left) and cortical actin (right) in NIH 3T3 MDR1 G185 cells. Bar 20 μm.

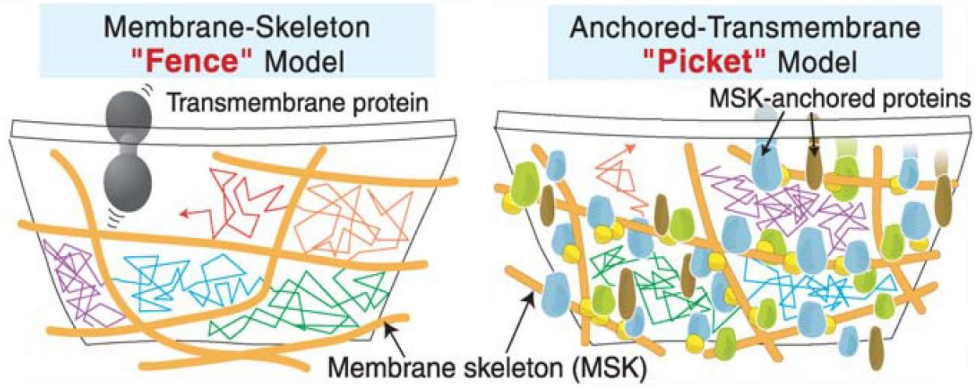

Figure 4. The models of the membrane-skeleton "fence" (left) and the anchored-transmembrane protein "pickets" (right) (Kusumi, et. al. 2005)

Two models have been described by Kusumi et.al. (14), i.e. the membrane-skeleton fence model, and the anchored-transmembrane protein picket model. According to these models the plasma membrane of the cell is a partitioned fluid membrane divided into "compartments" by the membrane skeleton (cortical actin). The transmembrane proteins can move freely only inside the "fence", or alternatively, these proteins are anchored to the membrane skeleton in case of the picket model (Fig. 4). In both cases the diffusion coefficients (represented with color arrows) of the free proteins and lipids differ in different "compartments". This was measured using single particle tracking (SPT) techniques and could explain why diffusion coefficients in the plasma membrane of cells are considerably smaller than those in artificial membranes.

Having thus depicted the stage at which ABC transporters perform, we will next describe systems and technology that serve in clarifying their distinct roles, including their mechanisms of action.

II. How does one study ABC transporters?

II.A. *In vivo* animal models

Animal models are still necessary tools in the drug development process. In today's age of polytherapy, it is impossible to avoid concomitant administration of two or more drugs. As a result, drug-drug interactions have become a common clinical problem and are a major cause for several adverse drug reactions. Pharmacokinetic drug-drug interactions occur when more than one drug shares a common clearance pathway involving a drug-metabolizing enzyme or transporter. On the other hand, pharmacodynamic drug-drug interactions occur when two drugs share a common pharmacologic mechanism of action. In general, adverse drug reactions are often associated with drugs able to induce or inhibit drug-metabolizing enzymes or transporters.

Although several methods have been published describing *in vitro* prediction of drug interactions, one has to accept that *in vitro* systems are not dynamic and often fail to predict drug-drug interactions. For example, it is possible that other metabolic pathways and

pharmacokinetic processes might also be altered by the inhibitor or may provide an escape from the pathway inhibited by the inhibitor. It is also likely that the real inhibitor concentration cannot be predicted based on plasma concentrations alone, especially if the inhibitor accumulates in the liver because of active transport (15). The application of *in vitro* and *in vivo* experiment should support each other. Moreover, the increasing availability of transgenic (humanized) rodent models will further enable the bridging of *in vitro* and *in vivo* data pre-clinically (16).

In the following we will describe two examples from today's wide range of *in vivo* models.

Over the last decade *in viv*o microdialysis (MD) has been increasingly applied to monitor drug distribution at peripheral tissue sites and changes in the chemistry of the extracellular space in living tissue or the penetration of various agents across the blood brain barrier (17,18). These techniques can be used for measuring drug and metabolite concentrations in the interstitial space in brain and peripheral tissues including blood or measuring concentrations of many analytes associated with tissue damage in the CNS and in various organs (19).

Biliary excretion studies are also possible in bile duct cannulated rats to study the excretion of test compounds into the bile or the effect of the test compound on the excretion of other (physiological) compounds (e.g. bile salts). The interaction with the ABC transporters Mrp2, Bsep and Bcrp will be detected as the modulation of biliary elimination of the respective reporter substrate (^{3}H-Estradiol-17β-D-glucuronide, ^{3}H-taurocholate and ^{3}H-Estrone-3-sulfate, respectively) in male Wistar rats (20).

II.B. Cell-based assay systems

Cell-based assay systems can be used in drug discovery and research to identify substrates and inhibitors for individual transporters. In addition, they can be used for studies to assess transport mechanism or transporter-based drug-drug interactions.

One application is the vectorial transport system using polarized cell lines, either with or without recombinant transporters in single- and double-transfected cell lines. In this system flux can be measured in two directions (apical-to-basolateral and vice versa) (4).

For example drug transport across the small intestine or the blood–brain barrier can be modeled in this way. Cultured cell lines, like HeLa, Mouse embryonic fibroblast (3T3) and Baby hamster kidney (BHK) or cell monolayers such as MDCK, HEK293, LLC-PK1 and Cho cells, are used for overexpression of recombinant transporters, which includes both uptake and efflux transporters.

Efflux assay

Dye efflux assays have proven their useful role in screening for compounds that inhibit the activity of ABC transporters, and that are likely capable of overcoming multidrug resistance. These assays are widely used to measure ABC transporter efflux activity in cell-based system. The cells of choice are usually loaded with small fluorescent molecules, such as 5-carboxyfluorescein diacetate (CFDA) and rhodamine 123 (R123) that serve as substrates for the transporters MRP1 and Pgp, respectively. This is done at 4-10 ºC, when ABC transporters are virtually inactive. The cells are then transferred to 37 ºC to al low efflux for various incubation times. Finally, after a cold inhibition step the remaining fluorescence is measured by flow cytometry. One should be careful to include a control with a specific transporter inhibitor to exclude the possibility of substrate leakage.

Calcein assay

The calcein assay is also a dye efflux assay, but in contrast to the above mentioned assays, this assay does not start with loading the cells. Calcein-AM is a non-fluorescent, hydrophobic acetoxy methylester (AM) form of the free calcein, which is fluorescent and hydrophilic. Calcein-AM is an excellent substrate for MRP1 and Pgp, and normally cells expressing one of these two transporters pump out calcein-AM rapidly. Only a very small amount will remain inside the cell, where esterases convert it to free calcein, which is a very poor substrate for the transporters. Over time, a slow accumulation occurs and a slow increase of the fluorescence intensity slope can be observed (R_{con}). When inhibited by e.g. cylosporin A, the transporters are inactive, so the intracellular calcein-AM (and free calcein) accumulation is increasing (R_{inh}). As a function of time the two different fluorescence intensity slopes are measured and a quantitative measure of transport activity,

the dimensionless multidrug activity factor (MAF), can be calculated accordingly to the formula: $MAF=[(R_{inh} - R_{con})/R_{inh}]x100$.

This assay is considered as the inhibition mode of dye assays, because substrates compete with the reporter substrate calcein-AM, thus increasing the calcein accumulation. The advantages of this method are that it is easy to perform, calcein has a bright green fluorescence, it is practically insensitive to changes of pH, Ca^{2+} or Mg^{2+}, and it can be applied as a high throughput method. BCRP related studies can be performed with the Hoechst assay using BCRP expressing cells and Hoechst33342 as reporter substrate.

II.C. Membrane-based assay systems

In addition to animal and cell models one can use membranes for example from mammalian or insect cells to study the ABC transporter function. A widely used approach relies on the isolation of the plasma membrane of cells, containing the transporter of interest. In most cases the cells are transfected with human transporter genes. Not just mammalian cell lines, but also insect cell lines are used. For example, by using membranes from Sf9 (insect) cells, it was demonstrated for the first time that Pgp displays ATPase activity (21).

ATPase assay

The ATPase assay is widely used as a screening tool in the pharmaceutical industry to detect drug-transporter interactions. ABC transporters use ATP as an energy source to transport substrates across cell membranes. The ATP hydrolysis yields inorganic phosphate (Pi), and the ATPase activity is detected as the amount of inorganic phosphate released by the enzyme by a colorimetric reaction. It is important to note that since the Pi is a by-product of transport of substrates, the assay thus relies on the assumption that the amount of Pi liberated by the transporter is considered to be proportional to the natural transporter activity.

The commonly used membrane preparations (vesicles), used for measuring ATPase dependent transporter activitity, contain other ATPases as well and it is important to

distinguish this background ATPase activity from the specific ABC transporter related ATPase activity. Thus a control with natrium-vanadate (Na_3VO_4) is part of the procedure, which effectively inhibits ABC transporters, thereby allowing determination of the transporter-dependent ATPase activity to the total ATPase activity. In this manner the activity of the transporters is correctly measured as the vanadate sensitive ATPase activity.

The assay has activation and inhibition modes. In the activation mode the interaction of potential substrates of the transporter can be measured. The activity measured by the compound is often represented on a scale, where 0 % is defined as the vanadate sensitive basal ATPase activity, and 100 % as the maximum vanadate sensitive activity observed in the presence of a well-known activator. In this case the compound is stimulating the ATPase activity of the transporter, and the activity measured is significantly higher than the basal activity of the transporter.

However one should be careful, because not every substrate can be measured in this way. There are slowly transported substrates and inhibitors that do not stimulate ATPase activity, and which can only be measured with the inhibition mode of the assay. In this case, the modulation of the maximal vanadate sensitive ATPase activity of the transporter by a well-known substrate is measured. Slowly transported compounds will compete with substrates, thereby inhibiting the transporter ATPase activity (22). This inhibition mode cannot distinguish between slowly transported substrates and inhibitors. However, it will indicate interactions that are not detected in the activation assay. A typical example of such interactions is cyclosporin A, which is commonly used as inhibitor. Although cyclosporin A is transported by Pgp (23), modulation of the ATPase activity can only be detected in the inhibition mode of the ATPase assay.

For proper evaluation of the ATPase assay, several considerations should be taken into account. First, unlike other primary transporters, ABC transporters can show uncoupled basal ATPase activity (24; see section II.E.). The basal activity is nevertheless calculated in the assay. Secondly, the ATPase assay measures only those ABC transporter molecules that are in the inside-out configuration. It should be noted that during the membrane vesicle isolation procedure both right-side-out and inside-out vesicles will be formed (see details below). Finally, when ATPase activity is measured at multiple substrate

concentrations a ‘bell-shaped’ curve is usually obtained. The ATPase activity initially increases with increasing substrate concentration, but subsequently decreases at higher concentrations. The currently accepted hypothesis for this phenomenon is a two-binding-site model (see section II.E.). For proper interpretation, it is therefore important to measure interactions of drugs over a wide range of concentrations (25).

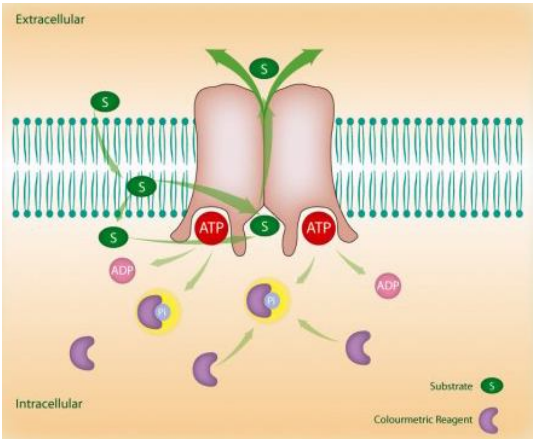

Figure 5. Schematic model of the ATPase assay (Solvo.com)

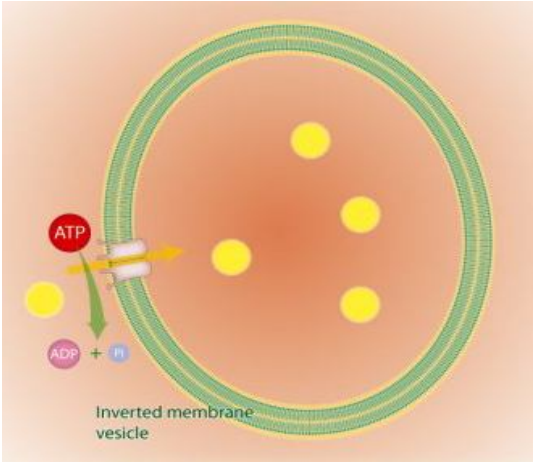

Figure 6. Schematic model of the vesicular transport assay (Solvo.com)

Vesicular transport assay

A major, obvious advantage of a vesicular transport assay is that it provides a direct measurement of substrate transport. Thus, whereas in the ATPase assay the amount of inorganic phosphate is measured, which is considered to be proportional to the transport, in the vesicular transport assay the actual transported amount of substrate is measured.

Membrane vesicle preparations always contain a mixture of so called right-side-out and inside-out vesicles. In case of inside-out vesicles the transporter's ATP -and substrate binding sites are facing to the outside, and the transported substrate will be pumped into the vesicles (Fig. 6). It is important to mention that the inside-out vesicle content is not necessarily 50 %, but more often only 20-30 %. After allowing temperature and ATP dependent transport of substrates into the vesicles, a rapid filtration step is done to halt the transport process, which is followed by ice cold washing to remove residual assay buffer with the remaining substrate. The control without ATP is important in order to determine free diffusion and membrane binding of the substrate. The amount of substrate can be determined by high pressure liquid chromatography (HPLC), liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), but also radiolabeled and fluorescent compounds can be applied.

Vesicular transport measurements can be used for the kinetic analysis of the biochemical parameters of the transport, yielding K_m and V_{max} values. Unfortunately, compounds with medium to high passive permeability cannot be measured because they are not retained inside the vesicles. This assay has also an inhibition or indirect mode. In this case the transport of a known substrate (reporter substrate) is measured in the presence of the test compound. This setup is suitable for the detection of possible drug–drug interactions and also high permeability compounds can be tested. However, it cannot provide information as to whether the compound tested is an inhibitor or a substrate of the transporter (25).

II.D. Inside-out vesicle ratio determination

Nanomedicines, drug delivery, and drug-transporter interactions are good examples of technologies in which vesicles derived from plasma membranes of cells are used. Interestingly, with most isolation procedures not all of the vesicles retain their native (right-side-out) configuration, and the ratio of right-side-out (RO) to inside-out (IO) membranes varies among cell types and different methods. Many examined parameters in these membranes depend critically on the orientation of the membrane vesicles. Knowledge of the sidedness of the preparation is thus essential, for example when comparing ABC transporter activity measurement in different systems (e.g. two preparations, two expression systems). By measuring vectorial transport in vesicle systems, only proteins located in inside-out vesicles will be activated. Do all samples have the same inside-out vesicle content? What happens after one or more freeze-thaw cycles? Obviously, results can no longer be compared to each other when the inside-out vesicle content may differ. Also the vesicle preparation methods used could be different. Hence, proper sidedness information of vesicles is therefore indispensable.

A typical feature of biological membranes is the asymmetric arrangement of protein molecules across the lipid bilayer. Some molecules are exclusively located on either the extracellular or the cytoplasmic side of the membrane. Accessibility of these molecules can be quantified, and this serves as the basis for sidedness determination methods, from what a few are described here:

Based on antibody

Antibody-specific methods can be used if the antibody binds exclusively to an epitope localized at a specific side of th**e** protein. In the case of ABC transporters, the antibody UIC2 binds to an external epitope of Pgp, whereas antibodies against MRP1 bind to internal epitopes. Such antibodies can be coupled to a column or magnetic beads (SIMAG-hydroxyl, 26), which can thus be used to separate IO and RO vesicles. Assuming that IO and RO vesicle size and protein content are equally distributed, the IO ratio could be calculated with the help of Western Blot or protein content analysis.

His-tag

In a similar way, if the constructed protein in question contains a HIS-tag e.g. in the cytoplasmic domain, the tag can be used to selectively bind inside-out vesicles to Ni-NTA agarose beads, and thus serve as the basis for sidedness measurement and/or isolation of a specific vesicle population.

Sialic acid - Concanavalin A-Sepharose

Resch et. al. (27) used a method, where they described the binding of membrane fractions to concanavalin A-sepharose. The binding is possible because of the sialic acid residues, which are exclusively localized in the outer monolayer of the plasma membrane. Thus, only right-side-out vesicles are bound. Kondo (28) took advantage of this principle by recognizing that there are no glycoproteins on the internal side of erythrocyte membranes. Thus, inside-out vesicles do not interact with concanavalin A.

5`nucleotidase

Determination of the inside-out vesicle ratio with 5`nucleotidase activity is based on a method, originally described in 1968 (29,30,31), but modified in our lab (see Chapter 3). Adenosine-5'-monophosphate (AMP) is the substrate of 5`nucleotidase and the inorganic phosphate released by this ectoenzyme is measured with the malachite green system (32). 4 conditions are necessary to determine the vesicle sidedness: A: measurement with AMP and Triton X-100 provides the maximum activity after the vesicles are disrupted. B: samples with AMP but without Triton X-100 give the activity of 5`nucleotidase in right-side-out vesicles. C: a control without AMP but with Triton X-100 shows all the background phosphate present in the system. D: the last negative control without AMP and without Triton X-100 indicates the background phosphate in the assay buffer. A-C yields the total activity of the enzyme; B-D shows the activity of 5`-nucleotidase in the right-side-out vesicles only. The percentage of inside-out vesicles compared to the total amount is calculated as [[(A-C)-(B-D)]/(A-C)]x100%. According to our experience it is necessary to measure condition C, to show the inorganic phosphate located in the vesicles. At this nanomolar range of phosphate it can be a considerable amount.

Based on	Name		What is measured	References
A. Protein of interest	A.1.	Antibody	Protein or WB band	26
	A.2.	His-tag	Protein or WB band	26
B. Other indicators	B.1.	Sialic acid	Protein or WB band	27, 28
			Sialic acid amount	33,34,35,36
	B.2.	Actin	DNase I inhibition	37
	B.3.	Enzyme based	Na^+/K^+-ATPase (Pi)	38,39,40
			5'-nucleotidase (Pi)	29,30,31,32
			H^+-ATPase pH change and (Pi)	41,42,43
			NADHcytochrome c oxidoreductase	33
			GAPDH	33
			Acetylcholinesterase	33

Table 2. Summary of the methods used for inside-out vesicle ratio determination

It is important to mention an alternative vesicle preparation method, the so called One Shot cell disrupter (44,45). This machine uses 400 bar pressure to force a cell sample through a small fixed orifice at high speed. By transferring the cells from a region of high pressure to one of low pressure, the cells are disrupted. Interestingly, it has been claimed that after the cells are disrupted, at least 90 % of the produced vesicles consist of inside-out vesicles. Because in the ATPase and vesicular transport assays only the IO vesicles are actively contributing, only by changing the vesicle preparation technique, it would be possible to prepare vesicles with ~3 times higher vesicular transport or ATPase activity and this is key to have an improved signal to noise ratio and more economic products.

II.E. Structural studies

Obviously, studies as described above have provided a wealth of insight into the functional activities of membrane transporters and ABC transporters in particular. Nevertheless, to grasp these functional aspects on the molecular level in the realm of biophysics, knowledge of the atomic structure of the ABC protein is imperative. Clearly, such insight can provide

answers to questions such as those of substrate binding sites and molecular mechanisms of transport-driving intramolecular changes within the protein structure. Importantly, such knowledge is crucial to knowledge-based discovery of novel drugs that can modulate the transporter.

This kind of structural studies may also shed light on the previously mentioned two-binding-site model. According to the current hypothesis the transporter has two distinct binding site for the substrate: one activating site with high affinity, localized in the cytoplasmic or transmembrane domains, and another inhibitory, low affinity site, probably in the extracellular domains. The relative affinity of the two binding sites is “normally” very different for the different substrates, and Michaelis-Menten type curves are observed in kinetic studies. However, if the binding affinity constants are more close to each other, the observed curve is bell-shaped.

The prevalent technique in membrane protein structure determination is X-ray crystallography of three-dimensional crystals. After decades of technical improvement the work has become considerably faster, thanks to developments in molecular biology and microfocus X-ray diffraction. The basis for success is a very pure protein sample. The two most critical factors that influence the purity level are the level of expression of the protein, and the choice of detergent, used to solubilize and purify the protein (46). Many membrane proteins cannot be purified using detergent solubilization because they denature and often aggregate. Many different detergents have to be tested, as the outcome relies on a trial-and-error approach.

At the level of protein expression, major advancements were made by molecular biology approaches, and the discovery of green fluorescent protein (GFP). When a membrane protein is fused N-terminally to GFP and folded properly, the GFP barrel will be synthesized as a fluorescent, sodium dodecyl sulphate (SDS)-resistant moiety. However when the protein biosynthesis is incorrect, GFP is misfolded, making the whole construct SDS-sensitive. The two conformations can be readily discriminated by Western-blot and/or immunoblots (47).

The molecular structure of Pgp was published in 1997 (48), and was based on 2D cryo-EM at 25 Å resolution. This technique revealed a large ‘aqueous chamber’ in the

transmembrane part of the protein, far away from the NBDs (Fig. 1, Fig.7). There is an 'opening' in the plane of the cell membrane leading into the chamber, suggesting that substrates can be directly accepted from the lipid bilayer (49,50).

The existence of such a 'chamber' within the bilayer supported a flippase model for ABC transporters. It was suggested that ligands might interact with binding sites that are located within the membrane. This is in agreement with the notion that the non-fluorescent calcein-AM is effluxed by Pgp before being converted to fluorescent calcein by cytoplasmic esterases.

The ligand-binding 'chamber' inside Pgp has a volume of approximately 5-6000 $Å^3$ (5-6 nm^3). The 'chamber', formed by the transmembrane domains, is exposed to the cytoplasm, and contains a laterally opening gate that is accessible to the inner leaflet of the membrane (Fig. 7; cover of this thesis). During transport, this binding 'chamber' is alternately exposed to the inside and the outside surface of the membrane. The transport process can be separated into four basic steps: (1) A substrate enters the binding 'chamber' from the inner membrane leaflet through the lateral gate between two TMDs or directly from the cytoplasm and binds the high-affinity ligand-binding site. (2) The TMDs undergo a conformational switch after binding and/or hydrolysis of ATP. They close the binding pocket to the cytoplasmic side of the membrane and open it to the extracellular side. The binding site is thought to reduce its affinity for the substrate by decreasing favorable intermolecular contacts, thereby increasing the off-rate. (3) The substrate is released from the 'chamber' into the outer leaflet of the membrane and/or extracellular environment. (4) After dissociation of the substrate, the TMDs reset to the cytoplasm facing high-affinity state described in (1). It is important to mention that the transport cycle most likely involves multiple intermediate states (51).

The affinity of Pgp for ATP increases when substrates bind in step 1 and this facilitates the second ATP binding. If there is no drug binding, the second ATP rarely binds, because the affinity remains low. However this happens with low rate and initiates the so called uncoupled basal ATPase activity (24).

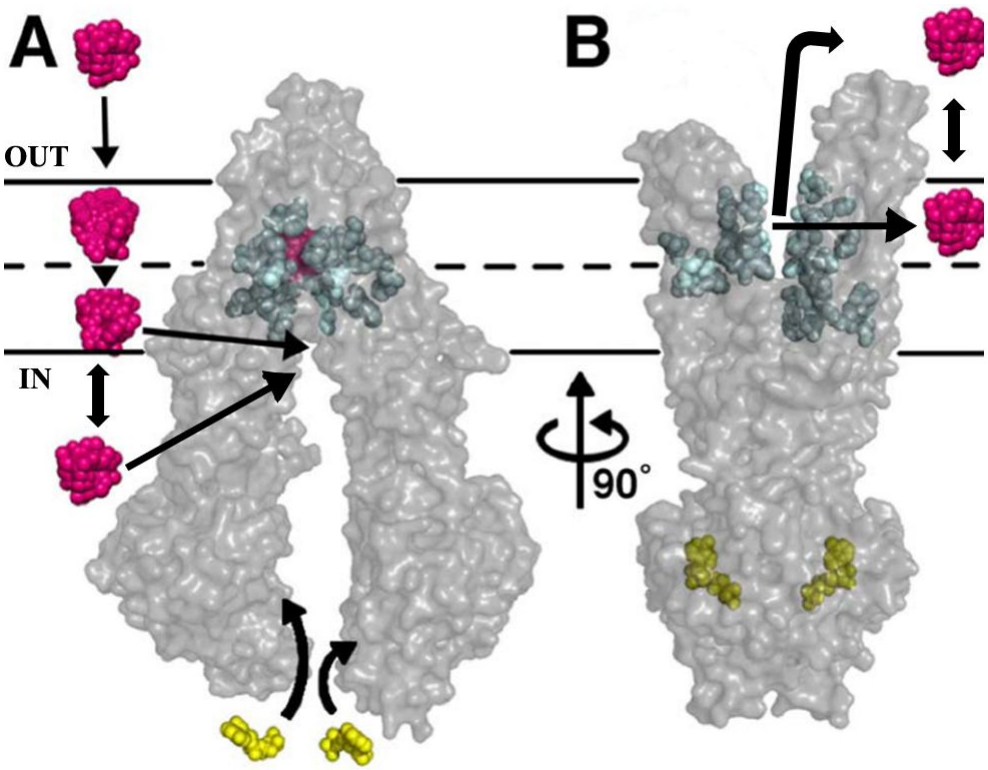

Figure 7. Model for ligand efflux by Pgp
In **panel A** the substrate (magenta) enters the membrane inner leaflet and binds (possibly through the cytoplasm) the internal drug-binding pocket through an open gate. The residues in the drug binding pocket (cyan spheres) interact with the substrate in this inward facing conformation. In **panel B** ATP (yellow) binds to the NBDs causing a conformational change that opens the drug-binding site and, consequently, the substrate is released to the outer leaflet/extracellular space. (adopted from Chang et. al. 2009)

III. How do we study ABC transporters? – Scope of this thesis

The aim of this Thesis is to study ABC transporter activity as a function of their molecular environment (e.g. raft or non-raft localization) in the lateral plane of the membrane. These studies will be carried out with both intact cells and membrane vesicles, allowing a greater

degree of experimental flexibility and hence a better appreciation of transporter activity. The general concept on which this study is based relies on the notion that ABC transporters function in the context of a network of molecules, involving lateral and transverse aspects of the membrane in which they are embedded. Laterally, ABC transporters are at least partly localized in lipid raft domains, rich in (glyco)sphingolipids and cholesterol. Transversely, lipid rafts are connecting to the cortical actin network, beneath the plasma membrane. ABC transporters may connect to actin via these lipid rafts or alternatively directly via actin-binding proteins.

Following a more general and experimental introduction into the topic of ABC transporters in **Chapter 1**, we review potential interactions of ABC proteins with lipid rafts and the actin cytoskeleton in **Chapter 2**.

In **Chapter 3**, the cholesterol dependence of MRP1 is investigated. The cholesterol content of the plasma membrane was decreased or increased, followed by measurement of MRP1 activity and analysis of its extent of lipid raft association. Activity was measured in both intact cells and membrane vesicles.

In **Chapter 4** we describe effects of depletion of glycosphingolipids on MRP1 activity, as accomplished by blocking glycosphingolipid biosynthesis. Particular attention is paid to the lipid raft association of the transporter. Detailed kinetic analyses are carried out in studies with membrane vesicles in order to determine the extent to which parameters, like number of MRP1 transporters and intrinsic substrate affinity, are modulated as a function of glycosphingolipid depletion.

In **Chapter 5**, we extend previous studies on MRP1-actin interactions to the closely related family members MRP2, MRP3 and MRP5. We investigate their localization using confocal microscope and lipid raft analysis, furthermore their efflux activities are compared in context of actin disruption.

Chapter 6 extends these studies to the realm of Pgp. Pgp activity and its association to lipid rafts is studied after disruption of cortical actin in a mouse and a human cell line. Also electron microscopy is used to visualize the different membrane alterations and surface labeling of Pgp to measure the possible internalization.

The findings are summarized in **Chapter 7**. We also discuss the relevance of our results with respect to the literature with an emphasis on the mutual beneficial use of intact cells and membrane vesicles for the study of ABC transporters.

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Chapter 2

Are lipid rafts involved in ABC transporter-mediated drug resistance of tumor cells?

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Chapter 2

Abstract

Since their discovery, lipid rafts have been implicated in several cellular functions, including protein transport in polarized cells and signal transduction. Also in multidrug resistance lipid rafts may be important with regard to the localization of ABC transporters in these membrane domains. This specific localization may support the activity of these ABC transporters as drug efflux pumps and this raises important questions regarding the dependence on lipid raft constituents. In this respect, two lipid classes immediately come into play, as both sphingolipids and sterols are generally assumed to be important in the generation and maintenance of lipid rafts. Apart from lateral interactions with lipids in the membrane bilayer, one can also envision that transverse interactions with respect to the membrane bilayer play a role in positioning and function of ABC transporters. Indeed, some evidence exists for a role of the actin cytoskeleton in stabilizing the position of ABC transporters in a certain membrane area. ABC transporters may be directly linked to the actin cytoskeleton, or indirectly via lipid rafts. In this review, we will evaluate whether ABC transporters are dependent on a particular membrane environment for their function and which of the lipid raft constituents appear to be essential for this dependency.

Introduction

There are many mechanisms by which tumor cells can become resistant to drugs. Some of these have as a consequence that the tumor cells do not only become resistant to a single, previously encountered drug, but rather acquire resistance to a wide variety of drugs. This phenomenon is called multidrug resistance and is based on a variety of underlying molecular mechanisms. One of these is the over expression of ABC transporters that remove drugs from the intracellular environment, after these drugs have gained access to the cell by diffusion through the plasma membrane. These ABC transporters reside mostly in the plasma membrane and appear to be influenced in their function by their local environment. In this respect, lipids come into play and ABC transporters have for long been known to depend on a certain lipid environment for optimal function. The question arose whether lipid rafts, i.e. domains in the plane of the membrane usually enriched in cholesterol and sphingolipids, are involved in localizing ABC transporters in a specific lipid environment to keep them functional. This hypothesis was fueled by a number of observations indicating that ABC transporters are localized in DRMs and affected in their function by manipulation of cholesterol levels in these membrane domains. On the other hand, several ABC transporters have been shown to associate with the actin cytoskeleton. In the light of the notion that the actin cytoskeleton associates with lipid rafts and possibly is involved in lipid raft stability as well as dynamics, a challenging hypothesis unfolds: Could it be that the actin cytoskeleton is involved in stabilizing ABC transporters in a specific membrane environment?

This review deals with the question whether MDR relevant ABC transporters are localized in lipid rafts to acquire optimal function and whether their localization in these domains is stabilized by surrounding raft lipids on the one hand and the actin cytoskeleton on the other. We will focus on ABC transporters with an established function in tumor cell drug resistance, i.e. Pgp, MRP1 or BCRP. It is clear that other lipid-mediated mechanisms can operate to modulate ABC transporter-mediated MDR. For example, the expression of ABC transporters is regulated by cellular cholesterol (1) and also changes upon modulation of sphingolipid metabolism (2). Obviously, the level of ABC transporter expression will

impact on the total cellular drug efflux activity. Conversely, ABC transporters can modulate lipids, for example when lipids are substrates for the ABC transporter and thus are flipped between the two monolayers of the plasma membrane. There is ample evidence that this can occur for cholesterol (3), while a flippase function for native sphingolipids is under debate. Although highly relevant to combat the process of MDR, these issues are beyond the scope of this review.

Lipid rafts

The lipid raft hypothesis was originally based on observations in sterol-containing model membranes, in which liquid-ordered (10) phase domains were shown to exist. Similar domains were proposed to exist in cell membranes, especially in the plasma membrane (4). They appear to be small in size, but together may constitute a relatively large fraction of the plasma membrane (5,6). These sub domains of the plasma membrane are considered to contain high concentrations of cholesterol and sphingolipids. The high content of sphingolipids gave rise to two different models for lipid raft formation in cells. The first model points out the importance of the relative long length and high saturation of the acyl chains of sphingolipids for lipid raft formation. This allows close packing of the lipids resulting in a high melting temperature (Tm). The self-aggregates of sphingolipids form a separate phase that is less fluid (liquid-ordered) than the bulk liquid-disordered phospholipids. Cholesterol is recruited to these aggregates, due to its ability to pack tightly with lipids of high Tm (7,8). According to the second model, lipid rafts are primarily clusters of sphingolipids held together through hydrogen-bonding between glycosphingolipid head groups. Cholesterol fills up the gaps between the acyl chains of the glycosphingolipids with their bulky-heads (9).

The tight packing of sphingolipids in these proposed lipid rafts could be responsible for their insolubility in nonionic detergents at low temperatures. Indeed, lipid rafts have been defined operationally by their low density and insolubility in cold 1% Triton X-100 (8,10). In addition, a wide variety of detergents other than Triton X-100 have been used to isolate low-density detergent insoluble membrane fractions (11-14). When detergent is used to isolate membrane domains, we refer to these membrane domains as detergent-resistant

membranes. DRMs isolated with different detergents display significant differences in protein and lipid composition, which suggests that different membrane domains are isolated. Indeed, several studies indicate that different liquid-ordered domains co-exist in the plasma membrane (15-17).

A specific subclass of lipid rafts is caveolae, which have mainly the same lipid composition, but can be distinguished by the presence of the cholesterol binding protein caveolin. In contrast to other lipid rafts, caveolae are the only ones that can be identified morphologically. Caveolae were known initially for their ability to transport molecules across endothelial cells, but many more functions have been and are still discovered (18,19). Caveolae are also known as membrane specializations where vesiculation can occur, giving rise to the formation of endocytic vesicles that carry material into the cell interior (20). In this respect caveolae resemble clathrin-coated pits, which are involved in the clathrin-mediated endocytic uptake pathway, by which many cell surface receptors are internalized.

The use of detergents to isolate lipid rafts has been subject of extensive discussion and criticism (21-23). It has been suggested that detergents induce artifacts, e.g. reorganization of molecules during the process of lipid raft isolation. Fortunately, detergent-free preparations of membrane domains have also been developed (24-26), avoiding the potential detergent-induced reorganization artifacts. Clearly, the discussion concerning the actual existence of lipid rafts in intact cells at physiological temperatures will continue and should be fueled by future data obtained by using novel and improved methodology for studying lipid rafts. What seems to be a fair conclusion, irrespective of the actual existence of lipid rafts in cells, is that various proteins in cell membranes show an increased tendency to associate with those cell lipids that form liquid-ordered domains in model membranes. This property has been termed raftophilicity (4 and ref. 59 here in).

ABC transporters involved in multidrug resistance are associated with lipid rafts

Different proteins have been shown to be associated with DRMs, especially proteins that are involved in cell signaling, such as receptor tyrosine kinases like the PDGF receptor and G protein coupled receptors. Therefore lipid rafts are considered to play an important role

in cell signaling (10). In polarized cells lipid rafts are also believed to play an important role in the sorting of apical resident proteins. Glycosylphosphatidyl inositol (GPI) anchored proteins, like placental alkaline phosphatase (PLAP), were shown to be sorted to DRMs during transport to the apical surface (5,9).

One of the best characterized multidrug resistance mechanisms in tumor cells is the over expression of energy-dependent drug efflux proteins, which prevent intracellular drug accumulation. ABC transporters are primary active transporters which bind their substrate and move it through the membrane against a substrate gradient at the expense of ATP (27). The ABC transporter super family is a large family of proteins, which in humans consists of 48 members. With regard to tumor cell drug resistance the most important ABC transporters are Pgp or (28), MRP1 (29) and BCRP (29).

Early studies revealed that Pgp depends on its lipid environment for optimal functioning (30,31). Upon reconstitution in vesicles, Pgp was found to have a higher affinity for its substrates when the surrounding lipids are in gel phase rather than in liquid-crystalline phase (32). Moreover, Pgp-mediated ATP hydrolysis as well as drug transport rate remain high in the gel phase (33,34). A gel phase occurs when lipids have a high degree of saturation, which enables them to pack tightly. This is also an important characteristic of membrane microdomains such as caveolae and lipid rafts (7,35). Thus, Pgp function would be compatible with its localization in membrane microdomains. Accordingly, Lavie et al. (36) have shown for the first time the association of an ABC transporter protein with a detergent resistant membrane domain in intact cells. They observed that a substantial fraction of the Pgp pool was located in caveolin-1 containing Triton X-100 insoluble membrane domains in Pgp over expressing cells. Furthermore, caveolae and the caveolin-1 protein were up-regulated (36). Pgp and caveolin-1 were coimmunoprecipitated from Pgp over expressing CHRC5 Chinese hamster ovary cell lysates, which suggests that the two proteins physically interact (37). Later, this group showed that in endothelial cells from the blood-brain barrier, Pgp function was regulated by caveolin-1 through its phosphorylation state (38). However, the association of Pgp with caveolae has been challenged. In 2780AD human ovarian tumor cells, which do not express caveolin-1 and hence lack caveolae, Pgp was still located in DRMs. Pgp was highly

enriched in membrane domains defined by their insolubility in the non-ionic detergent Lubrol WX (11). Another study in the MDR Chinese hamster ovary cell line CHRB30 showed that Pgp was localized in intermediate density domains, which can be isolated using Brij-96. These domains were shown to be distinct from caveolae, as indicated by absence of microscopically detected colocalization and absence of coimmunoprecipitation of Pgp and caveolin-1. This suggests that Pgp does not interact directly with caveolin-1 in MDR Chinese hamster ovary cells. Moreover, the Pgp containing Brij-96 domains appeared to be distinct from classical lipid rafts, as indicated by a differential gradient distribution of Pgp and Yes kinase, a Src-family kinase used as classical lipid raft marker (39). The mutual relationships between Pgp and lipid rafts have been elegantly reviewed recently (1). The authors summarize studies of Pgp association to DRMs, which turns out to be variable, depending on the cell type and the type of detergent employed to isolate DRMs.

Also MRP1 has been associated with lipid rafts, although studies are much scarcer than those related to Pgp-lipid raft association. In HT29col human colonic tumor cells, MRP1 was enriched in membrane domains defined by their insolubility in the non-ionic detergent Lubrol WX. Although these cells do express caveolin-1, MRP1 did not appear to be localized in caveolae. MRP1 was not enriched in caveolin-1 containing Triton X-100-based DRMs. Moreover, MRP1 and caveolin-1 were dissociated on the basis of absence of microscopically detected colocalization and absence of coimmunoprecipitation (11). While MRP1 expression had increased dramatically during MDR acquisition, caveolin-1 expression remained unaltered relative to drug-sensitive cells. The differential insolubility of caveolin-1 and MRP1 in different detergents indicates an association with different membrane domains. Hence, it appears unlikely that caveolin-1 or caveolae play a significant role in the accommodation of MRP1 in HT29col cells. Recently, MRP1 was shown to be exclusively associated with light membrane fractions representing L0 phase membrane microdomains, as isolated using a detergent-free isolation procedure, in GLC4/ADR MDR small lung cancer cells (40). On the other hand, in another recent study the conclusion was reached that MRP1 is not associated to DRMs. This conclusion was based on a different gradient distribution of MRP1 compared to the DRM marker flotillin-1

after Triton X-100 isolation of DRMs and absence of colocalization of MRP1 with the lipid raft marker GM1 (41). However, these results can be reconciled with those of the aforementioned studies in the sense that MRP1 and in certain cell types also Pgp may not be associated with caveolae or classical lipid rafts, as defined by Triton X-100 isolation and co-distribution on gradients with markers such as Src-family kinases, caveolin-1, flotilin-1 and GM1. Rather, these ABC transporters appear to be associated with a novel type of lipid rafts, as defined by Lubrol WX or Brij-96 isolation. In addition, these domains may be isolated using detergent-free lipid raft isolation procedures (40). In this respect, we have recently obtained evidence for a partial localization of MRP1 in detergent-free lipid raft gradient fractions in both Neuro-2a murine neuroblastoma cells and MRP1 over expressing hamster BHK-MRP1 cells (Klappe K., Dijkhuis A.J., Hummel I., Boer A. de, Huls A.M., Dam A. van, Permentier H., Kroesen B.J., Sietsma H., and Kok J.W., unpublished observations).

Concerning BCRP, a recent study showed for the first time its association with lipid rafts in BCRP over expressing MDCKII canine kidney epithelial cells (42). This ABC transporter was to a large extent localized in DRM gradient fractions after Triton X-100 isolation and co-distributed with caveolin-1. Caveolin coimmunoprecipitated with BCRP, but BCRP did not coimmunoprecipitate with Caveolin. It was concluded that BCRP resides in caveolae and physically interacts with caveolin-1. It would be worthwhile to study DRM association of BCRP using other detergents or detergent-free lipid raft isolation procedures as well as other cell lines, to investigate whether it shows similar cell type and detergent dependent behavior as Pgp and MRP1.

ABC transporter function is affected by raft lipid manipulation

Lipid rafts are considered to be enriched in sphingolipids and sterols (see above) and these two lipid families will be the main subject of the following discussion. However, it should be noted that regarding total lipid mass of ABC transporter containing DRMs phospholipids actually constitute the major portion and these lipids should also be involved in studies on DRM association of proteins and modulation of their function/activity (43). Given the proposed properties of lipid rafts, it is quite surprising that virtually all studies regarding

localization and function of proteins in membrane domains rely solely on two criteria: The protein of interest 1) should be floating to "raft fractions" in density gradients after detergent or non-detergent isolation and 2) should be affected by cholesterol manipulation concerning its localization in raft fractions and preferably also its function/activity. Very few studies have made use of the other property, i.e. sphingolipid enrichment, to show that manipulation of sphingolipids indeed also affects the localization and function of lipid raft associated proteins (e.g. 44).

Cholesterol

Concerning cholesterol manipulation, the vast majority of studies relies on the use of cyclodextrins (e.g. methyl-β-cyclodextrin) to physically remove cholesterol from the membrane. In addition, cholesterol-loaded cyclodextrins can be used to replenish/supply cholesterol (or other sterols) to the membrane, which should lead to reversal of the effect accomplished by cholesterol depletion. Other tools, which have been used include cholesterol oxidase to chemically convert cholesterol to cholestenon, lacking the 3β-hydroxyl group which confers important biological functions to cholesterol, filipin treatment, which results in reorganization of membrane cholesterol and statins (e.g. lovastatin (LO)), which inhibit an early step in cholesterol biosynthesis and thereby decrease membrane cholesterol levels. In our view, these alternative ways to modulate the membrane cholesterol level and/or its localization should be more widely applied in conjunction with cyclodextrin treatment to provide evidence for - or to the contrary of - the involvement of cholesterol and lipid rafts in protein localization and function. This is especially relevant, since it has been clearly shown that cyclodextrins are not specific for cholesterol and also remove other lipids, such as phospholipids (45,46 and our own unpublished observations). In an elegant study Rouquette-Jazdanian et al. revaluated the role of DRM-associated cholesterol in T cell receptor signaling (46). They showed that methyl-β-cyclodextrin treatment extracts two pools of cholesterol, a fast ($t_{1/2}$ = 17 sec) pool which corresponds to DRM-associated cholesterol and a slow ($t_{1/2}$ =15 min) pool, which corresponds to cholesterol from membranes other than rafts. Depletion of the fast DRM-associated pool of cholesterol did not affect T cell receptor signaling, as evidenced by intact

DRM association and tyrosine phosphorylation of signaling proteins downstream of CD3. Depletion of the slow pool, on the other hand, did result in loss of signaling proteins from DRMs and inhibition of phosphorylation and thus affected T cell receptor signaling. These effects were not reversed by cholesterol replenishment and were attributed to the side effects of methyl-β-cyclodextrin treatment, which include loss of DRM-associated signaling proteins and loss of phospholipids, as well as plasma membrane depolarization, likely the result of permeabilization of the plasma membrane. In support of this view, cholesterol oxidase treatment leads to specific oxidation of DRM-associated cholesterol to cholestenon. The latter sterol moved out of the DRMs, but CD3 signaling was not affected.

Returning to ABC transporters, Troost et al. (47) have shown in porcine kidney epithelial cells over expressing the human MDR1 gene (L-MDR1 cells) that cholesterol depletion by methyl-β-cyclodextrin reduces Pgp activity with a concomitant complete shift of Pgp out of DRM fractions. Cholesterol repletion restored both DRM association of Pgp and Pgp activity, while cholesterol saturation even further increased Pgp activity. This study thus provides support for a link between lipid raft association of Pgp and its functional activity. In another study methyl-β-cyclodextrin was used to remove cholesterol and subsequently replenish cells with either cholesterol or ent-cholesterol, a synthetic enantiomer of cholesterol. Methyl-β-cyclodextrin treatment resulted in a shift of Pgp from low to high density membranes in NIH 3T3 cells, and this effect was reversed by both sterols. Pgp function, however, was not dependent on its localization in cholesterol-enriched membranes in these cells (48). Two studies by the group of Uekama used dimethyl-β-cyclodextrin and revealed that inhibition of Pgp function in Caco-2 cells by this compound could be (partly) attributed to release of Pgp from the membrane as a secondary effect upon cholesterol depletion in caveolae (49). Moreover, the effect of dimethyl-β-cyclodextrin might require extraction of phospholipids in addition to that of cholesterol (50). Finally, one study indeed compared the effects of various methods to manipulate membrane cholesterol and showed that methyl-β-cyclodextrin and filipin, but not cholesterol oxidase decreased Pgp activity in K562/ADR cells. Moreover, using detergent-free methodology, Pgp was not found in “light” membrane fractions, where lipid rafts are detected. This leads to the conclusion that Pgp is not localized in lipid rafts in these cells.

The effects of cholesterol depletion using methyl-β-cyclodextrin and filipin were attributed to an increase in membrane fluidity (51).

In conclusion, cholesterol depletion studies have not provided a definite answer as to whether Pgp association to lipid rafts is necessary to provide optimal function of this ABC transporter. Although some studies show a clear correlation between these two parameters, there are a number of factors that potentially contribute to altered Pgp activity after cholesterol depletion treatments (especially those based on the use of cyclodextrins), including altered membrane fluidity, release of Pgp from the membrane and release of other molecules, such as phospholipids.

Concerning the other two ABC transporters highlighted in this review, i.e. MRP1 and BCRP, cholesterol modulation studies are scarce. In MRP1 over expressing GLC4/ADR cells, MRP1 partially shifted to high-density membrane fractions upon methyl-β-cyclodextrin treatment concomitant with a decrease of its efflux function. This occurred only when cholesterol was depleted below 40% (40). We have obtained similar data in Neuro-2a murine neuroblastoma cells and BHK-MRP1 cells over expressing MRP1, showing correlation between a shift of MRP1 out of detergent-free lipid rafts and reduced MRP1-mediated efflux. However, cholesterol oxidase treatment resulted in a very efficient conversion to cholestenon but was without effect on both lipid raft localization and function of MRP1 (Klappe K., Hummel I., Mészáros P., Ercan C., Kroesen B.J., Sietsma H., and Kok J.W., unpublished observations). The conclusion so far seems to be that MRP1 function is coupled to lipid raft localization, but not to the cholesterol level. Concerning BCRP, it has been shown that cholesterol potentiates its function in a study which did not investigate its association to lipid rafts (52). In another study both BCRP localization in membrane domains – in particular caveolae – and modulation of its activity by cholesterol depletion (methyl-β-cyclodextrin) were shown, but whether these two phenomena are correlated was not investigated. Thus, in the case of BCRP, the importance of membrane domain localization for its function remains to be established.

Sphingolipids

It is clear that uncertainties remain with the current state of the art concerning the relevance of lipid raft localization for the function of ABC transporters. We think it is important to discover and develop other mechanisms and methods, respectively, that modulate the extent of lipid raft association of ABC transporters and to monitor the concomitant change in ABC transporter function. In this respect, it is worthwhile to investigate the effects of sphingolipid manipulation on the lipid raft localization and functional activity of ABC transporters. In one study in Pgp over expressing MDR1-MDCK cells, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was employed to deplete the cells of all glycosphingolipids by inhibition of the enzyme glucosylceramide synthase. Under these conditions, Pgp was shifted to higher density gradient fractions (53). PDMP affected Pgp function in MDR myeloid leukemia cells (54) and moderately in HepG2 hepatoma cells (55), but not in Pgp over expressing 2780AD ovarian tumor cells (56). In the latter studies (54-56) lipid raft association of Pgp was not investigated. Promising results were obtained in the MRP1 over expressing HT29col colon tumor cell line, which displayed a synchronous up-regulation of both MRP1 and glucosylceramide during culturing with gradually increasing colchicine concentrations. Moreover, both were up-regulated in DRMs. This suggested a role for glycolipids in MRP1 localization in DRMs and its functional activity. However, when glycolipids were depleted in these cells, MRP1 function was not affected (57). It is important to note that the use of PDMP is not without risk, because this inhibitor is known to have many side effects in cells (for an extensive discussion see ref. 58). Therefore, the much less toxic compounds of the iminosugar family, such as N-butyl-(galacto)deoxynojirimycin, are preferred inhibitors of glucosylceramide synthase. Using these compounds, glucosylceramide synthase function was dissociated from MDR (59). Moreover, it was shown that both the localization in DRMs and the efflux function of Pgp and MRP1 were not affected in spite of efficient depletion of glycolipids in human neuroblastoma cell lines SK-N-FI and SK-N-AS, respectively (60). Taken together, glycolipids in some cells appear to modulate Pgp, but are clearly not essential as they are without effect in other tumor cell lines. A lack of effect of glycolipid depletion on DRM localization of ABC transporters is not surprising, given that glycolipids – although

enriched in DRMs - do not appear to be essential for their formation. It was shown that glycolipid-deficient GM95 melanoma cells had similar amounts of DRMs compared to control cells. While the fluidity of the DRMs isolated from both cell lines was similar, glycosphingolipids in DRMs of GM95 cells had been substituted by sphingomyelin (61). Thus, inhibition of glucosylceramide synthase resulted in a compensatory cellular mechanism that restored the total sphingolipid pool. Moreover, inhibition of glucosylceramide has the disadvantage that it can cause accumulation of the precursor ceramide, which is well-known as a signaling molecule that can affect MDR related cell physiology, such as the tendency of cells to engage in apoptosis. Therefore, in order to circumvent both of these cellular adaptation/activation mechanisms the approach of depleting all sphingolipids comes into play. Many studies have been performed with myriocin (ISP-1), which inhibits serine palmitoyl transferase, the enzyme that catalyzes the first step of sphingolipid biosynthesis. However, to our knowledge no studies have been published using myriocin to investigate the role of sphingolipids in ABC transporter localization and function. We were able to efficiently deplete all sphingolipid classes in Neuro-2a as well as in BHK-MRP1 cells by using myriocin. Under these conditions, the localization of MRP1 in DRMs as well as in detergent-free lipid rafts was completely unaffected. In accordance, MRP1 function was equal to that of control cells (Klappe K., Dijkhuis A.J., Hummel I., Boer A., de Huls A.M., Dam A. van, Permentier H., Kroesen B.J., Sietsma H., and Kok J.W., unpublished observations). This leads to the conclusion that sphingolipids are not involved in localizing MRP1 to lipid rafts and are dispensable for MRP1 function. This also shows that ceramide is not involved in modulating MRP1-mediated MDR. These studies need to be extended to the localization and function of Pgp and BCRP with regard to their possible dependence on sphingolipids.

Phospholipids

Our studies revealed that Pgp and MRP1 were enriched in Lubrol-based DRMs in human tumor cell lines (11,43). When these DRMs were carefully analyzed, they were enriched in cholesterol and sphingolipids, the latter however to a lower extent than the sphingolipid enrichment in Triton X-100-based DRMs. In addition, Lubrol-based DRMs contained twice

the amount of protein and phospholipid compared to Triton X-100-based DRMs. Compared to Triton X-100-based DRMs, Lubrol-based DRMs were enriched in phosphatidylethanolamine and phosphatidylserine, which is quite compatible with the well-known dependence of Pgp and MRP1 on these aminophospholipids for their ATPase activity (62-65). We have proposed a hypothetical model in which ABC transporters and sphingolipids co-exist in layered rafts (66). The layered raft model has already been proposed in an elegant review by Pike (67) as one of three alternative models. All three models, including the homogeneous and heterogeneous raft models, could explain the variation in lipid and protein composition observed in rafts isolated by different protocols, using different detergents or detergent-free methods. These three models are not necessarily mutually exclusive and thus 'traditional' Triton X-100-based rafts may co-exist in cells with 'variant' rafts (e.g. Lubrol-based) as well as layered rafts. The layered rafts are composed of concentric layers of lipids ranging from a well ordered cholesterol- and sphingolipid-enriched core through less ordered regions that ultimately grade into the disordered structure of the bulk plasma membrane (67). In the MDR raft model, the Lubrol-based DRMs consist of a highly sphingolipid-enriched Triton X-100 insoluble core, surrounded by a Triton X-100 soluble region, which contains relatively high levels of specific aminophospholipids and harbors most of the ABC transporter molecules, which can optimally function in this lipid environment (66).

Proteins

In addition to lipids in the environment of ABC transporters, it is conceivable that specific lipid raft associated proteins influence the localization and activity of the transporters. In this respect, it has been shown in a MDR lymphoma cell line that anti-CD19 antibodies inhibited the interaction between Pgp and CD19. As a result, Pgp translocated out of lipid rafts and became inactive (68). This study shows that Pgp in these cells is active only when localized in lipid rafts, based on experiments that do not interfere with the lipid composition of the cells. Alternative to modulation of lipid rafts followed by analysis of ABC transporter localization and function, it is possible to directly assess the activity of the transporter in specific membrane environments. This was done in an elegant study by

Barakat et al. (69), who analyzed Pgp ATPase activity in DRM fractions and detergent-solubilized membrane fractions. The DRM associated Pgp displayed optimal ATPase activity, which was inhibited by orthovanadate and which was reversibly sensitive to cholesterol depletion; The Pgp in the detergent-solubilized membrane fractions displayed a lower ATPase activity, was less sensitive to orthovanadate and was not sensitive to cholesterol depletion and repletion. Also this study contributes to the hypothesis that Pgp is most active when localized in lipid rafts.

Another highly interesting group of proteins with which ABC transporters may interact is that of cytoskeleton, especially the actin cytoskeleton, and related binding proteins. We will now shift our focus from lateral ABC transporter – lipid/protein interactions in the plane of the membrane to transverse interactions with proteins that may (transiently) stabilize lipid rafts (Fig. 1). Before doing so, we will briefly review the concept of lipid raft – actin cytoskeleton interactions in general.

Lipid rafts and the actin cytoskeleton

On the one hand lipid rafts are often considered to be highly dynamic entities, which may arise and dissolve continuously. On the other hand, the actin cytoskeleton appears to have the potential to stabilize them, at least temporarily. In fact, actin dynamics may confer dynamic properties on lipid rafts, stabilizing them as separate domains upon actin binding while the lipid rafts are able to move laterally and coalesce when disconnected from the actin cytoskeleton. Using fluorescence resonance energy transfer (FRET) approach, Chichili and Rogers have shown that co-clustering of raft-associated donor and acceptor fluorescent probes is disrupted when Jurkat T cells are treated with latrunculin B, which inhibits polymerization of actin. The effect of latrunculin B is even larger that of filipin, which disrupts co-clustering of the lipid raft probes by cholesterol sequestration. Moreover, jasplakinolide, which promotes actin polymerization, enhances co-clustering. The authors conclude that the actin cytoskeleton acts in synergy with cholesterol to promote co-clustering between lipid raft resident proteins and that association of F-actin with the plasma membrane may participate in establishing lipid raft domains (70).

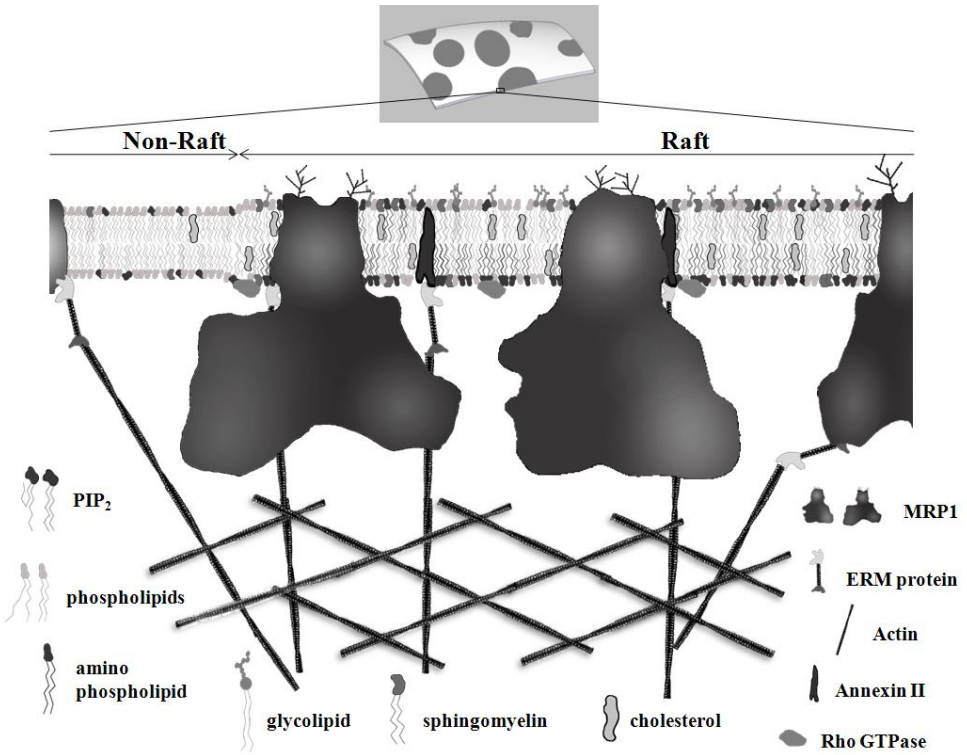

Figure 1. Model of potential membrane localizations of ABC transporters and their interactions with lipids and proteins in the membrane or adjacent to the membrane
ABC transporters, such as MRP1, are localized in lipid rafts, but are also found in the non-raft membrane. In lipid rafts they may interact with raft-specific lipids, such as cholesterol and sphingolipids, but also with other raft-associated proteins. In this way, ABC transporters are kept in a specific membrane location due to lateral interactions in the plane of the membrane. In addition, localization and stabilization of ABC transporters in a certain membrane area could be further established by interactions with the (cortical) actin cytoskeleton, which forms a dense network underlying the plasma membrane. This constitutes a transverse coupling system with respect to the plane of the membrane and may involve direct linkage of ABC transporters to actin via linker proteins, such as those of the ERM family, ezrin, radixin and moesin. Direct linkage to the cytoskeleton could occur for ABC transporters, irrespective of whether they are in lipid rafts or in non-raft membrane areas. Concerning lipid raft associated ABC transporter molecules, another possibility exists: They can be indirectly linked to the actin cytoskeleton via another integral membrane protein (hypothetically annexin II) that co-clusters with the ABC transporter in the same lipid raft and is tethered to actin. The ABC transporter would thus be stabilized in its localization by the actin cytoskeleton in the absence of a direct linkage to it. In this model, lipid rafts play an important role as the central entity which provides the optimal environment for ABC transporter function and which dynamically interacts with the underlying cortical actin network. Thus, the two axes of stabilization around the central lipid raft may together provide a network for correct localization and optimal function of the ABC transporter.

Levitan and Gooch (71) have published an intriguing review in which they discuss two lines of evidence in favor of a role of lipid rafts in membrane-cytoskeleton interactions. First, cytoskeletal regulatory molecules, such as phosphatidylinositol 4,5 bisphosphate (PIP2), Rho-type GTPases, and integrins, partition into lipid rafts. Although PIP2 has been shown to be enriched in lipid rafts (72), it should be noted that this issue is still controversial (73,74, for a detailed discussion see ref. 71). It is highly interesting, however, that cholesterol depletion by Methyl-β-cyclodextrin (M-β-CD) affected both the plasma membrane PIP2 level (50% decrease) and the actin cytoskeleton (fewer and thinner stress fibers; polymerization of cortical actin; refs. 71,75). PIP2, which is known for its actin modulating properties, thus emerges as a candidate molecular link between (the inner leaflet of) lipid rafts and the actin cytoskeleton. This also means that when one uses M-β-CD to manipulate lipid rafts, it should be taken into account that this may lead to simultaneous interference with the actin cytoskeleton. Effects of M-β-CD treatment on localization and function of specific proteins (e.g. ABC transporters) can therefore not be directly attributed to lipid raft disruption and actin involvement should be investigated as well. Second, several cytoskeletal proteins directly associate with lipid rafts. Proteomic and other methods of analysis of DRMs isolated from different cellular compartments of neutrophils as well as other cell types revealed the abundant presence of cytoskeletal proteins, including actin and actin-binding proteins, such supervillin, myosin, gelsolin and filamin (75-79). Based on these and other studies (80,81), Levitan and Gooch proposed three non-exclusive mechanisms by which lipid rafts may interact with the actin cytoskeleton, the intermediates being supervillin/myosin, filamin (in the case of caveolae) and ERM family proteins/annexin II, respectively (71).

Ezrin (cytovillin), radixin and moesin are closely related proteins belonging to the family of ERM proteins. They link actin filaments to the (plasma) membrane, either directly by binding to integral membrane proteins or indirectly via scaffolding proteins attached to integral membrane proteins (82). Soluble ERM proteins in the cytoplasm are ‘dormant’ in terms of their cross linking activity through intramolecular association between the N-terminal ERM association domain (N-ERMAD) and the C-terminal ERM association domain (C-ERMAD), masking membrane and F-actin binding sites. Activation

of the protein is thought to require binding of the protein to PIP2 and subsequent phosphorylation of a C-terminal threonine, which is also Rho-dependent. Following activation, the protein can bind to the membrane via its N-terminal FERM domain and to F-actin via its C-terminal tail.

A research area in which lipid raft-actin connections have received ample attention is that of lymphocyte migration and activation, including B-cell receptor (BCR) and T-cell receptor (TCR) signaling (83,84). First, cross linking of both BCR and TCR induces mobilization of the receptors into DRMs. Subsequently, many signaling partners are recruited and finally a large scale reorganization of lipid rafts occurs to form a macrodomain, the immunological synapse in the case of T-cells and large patches in case of B-cells (BCR capping). The actin cytoskeleton and actin-binding proteins are thought to play various roles in the latter process of macrodomain formation. Based on a quantitative proteomics approach and the use of constitutively active ezrin chimeras, it was shown that release of ezrin from lipid rafts triggers lipid raft dynamics during B-cell signaling (85). The hypothesis that emerged states that lipid rafts are dispersed on the cell surface of a resting B-cell and tethered to the actin cytoskeleton by phosphorylated ezrin, which is in the open and active conformation (see above). The actin cytoskeleton thus acts as raft stabilizer preventing coalescence of small lipid rafts. BCR stimulation leads to ezrin dephosphorylation and inactivation, which results in release of the lipid raft membrane from the actin cytoskeleton. This relieves the constraints on lipid raft dynamics and allows coalescence to proceed. In T-cells, formation of the large immunological synapse membrane domain involves co-clustering of TCR and CD28 in lipid rafts. CD28 binds and recruits filamin A and this allows actin-based coalescence of lipid rafts and their recruitment to the immunological synapse based on the formation of actin-filamin-CD28 complexes. Although we have focused here on lymphocytes, these are not the only cells in which lipid raft-actin associations have been described. This link is extended to other cell types varying from red cells and platelets to oligodendrocytes (86-88).

In conclusion, ample evidence has accumulated showing that lipid raft-actin cytoskeleton interactions exist and play important roles in cell physiology. It should be noted, however, that alternative views have been proposed based on novel and innovative

methodology. Lenne et al. have used a fluorescence correlation spectroscopy approach in live cells to establish the relative contribution of lipid-dependent (microdomain model) and cytoskeleton-based (meshwork model) processes in lateral compartmentalization of lipid raft markers and lateral organization of membranes. They arrive at the conclusion that compartmentalization of sphingolipid analogs and GPI-anchored proteins is cholesterol and sphingomyelin-dependent, but independent of the actin-based cytoskeleton organization (89). On the other hand, a study by Lillemaier et al. based on transmission electron microscopy of plasma membrane sheets showed that most or all plasma membrane-associated proteins are clustered in cholesterol-enriched domains that are separated by protein free and cholesterol-low membrane. They propose the 'protein island' model, where all membrane-associated proteins are clustered in protein islands, which can be subdivided into raft and non-raft islands. The protein islands are restricted in their lateral movement in the plane of the membrane due to connection to the actin cytoskeleton, which plays an important role in their formation and/or maintenance. Within the protein islands, high diffusion rates are possible (90).

ABC transporters and the cytoskeleton

Finally, we will address the question whether ABC transporters, notably Pgp, MRP1 and BCRP, are linked to the actin cytoskeleton (Fig. 1). Moreover, if they are connected to F-actin, it is important to establish whether this involves a direct link, mediated by one or more intermediate, actin-binding proteins and adaptors, such as those of the ERM family. ABC transporters could be linked in this fashion to F-actin, irrespective of their association with lipid rafts. Alternatively, raft-resident ABC transporters could be indirectly linked to the actin cytoskeleton, when they are co-clustered in the same lipid raft with other proteins that in turn are attached to F-actin. Thus, we can in theory discriminate five membrane pools of an ABC transporter: 1) non-raft associated and non-actin associated, 2) raft-associated, but non-actin associated, 3) raft-associated and indirectly linked to actin, 4) raft-associated and directly linked to actin, and 5) non-raft associated, but directly linked to actin (Fig. 2).

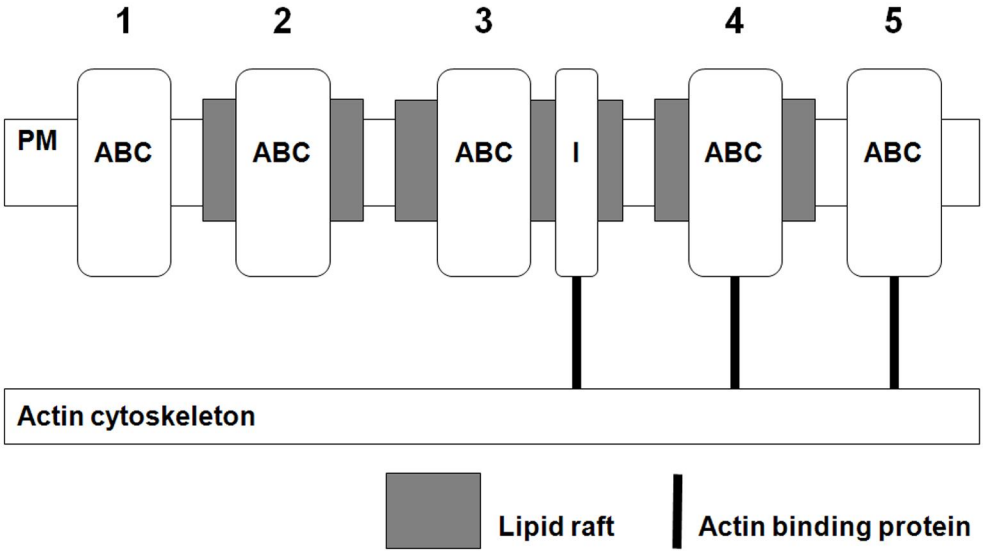

Figure 2: Scheme of potential membrane localizations of ABC transporters and their interactions with the actin cytoskeleton
Five possible membrane pools of an ABC transporter: 1) non-raft associated and non-actin associated, 2) raft-associated, but non-actin associated, 3) raft-associated and indirectly linked to actin, 4) raft-associated and directly linked to actin, and 5) non-raft associated, but directly linked to actin. PM = plasma membrane; I = integral membrane protein

Bacso et al. have developed an assay to measure the actin associated and indirectly actin linked pools of an ABC transporter, i.c. Pgp (91). This assay is based on two assumptions: 1) The detergent Nonidet P-40 dissolves all membrane proteins, except those that are directly linked to the actin cytoskeleton (i.e. it measures pools 4 and 5); 2) The detergent Triton-X-100 dissolves those proteins that are not in actin-linked DRMs and not directly linked to the actin cytoskeleton (i.e. it measures pools 3 to 5). With this assay pools 1 and 2, as well as pools 4 and 5, cannot be discriminated. By subtracting the Nonidet P40 values from the Triton X-100 values, pool 3 is obtained. It was observed that approximately 15% of Pgp molecules in MDR1 over expressing NIH 3T3 cells are directly linked to the actin cytoskeleton, while approximately 19% is in lipid rafts that are indirectly linked to F-actin (91). The authors speculate that direct linkage of Pgp to F-actin may involve CD44 and ERM family proteins.

Pgp-actin association through ERM family proteins has been shown in a MDR variant of a human T-lymphoblastoma cell line CEM-VBL100. Pgp interacted with ezrin, radixin and moesin as indicated by microscopical colocalization and coimmunoprecipitation. Interestingly, when all three ERM proteins were down-regulated using antisense oligonucleotides, actin-Pgp association was lost and Pgp redistributed in the cells. Concomitantly, the cells were sensitized to vinblastine, which accumulated due to reduction of Pgp-mediated drug efflux. These data indicate that the ERM protein-mediated link between Pgp and F-actin is functional in MDR (92). Raft association of Pgp, actin and ERM proteins were not analyzed in this study. Another clear example of ABC transporter-actin associations mediated by ERM proteins is the direct association of radixin with the carboxy-terminal cytoplasmic domain of human MRP2, which was discovered in Rdx-/- mice (93). Radixin deficiency in these mice results in loss of MRP2 from bile canalicular membranes, which leads to conjugated hyperbilirubinemia, a phenotype very similar to that of Dubin-Johnson syndrome characterized by mutations in ABCC2, encoding MRP2. Thus, the ERM protein radixin in liver cells is essential for the correct localization and the conjugated bilirubin secretion function of MRP2. The lack of radixin also results in deafness in mice, which is due to defective stereocilia in the inner and outer hair cells. The ERM proteins ezrin, radixin and moesin are considered to be functionally redundant and loss of one member of this family should not have drastic consequences. However, radixin is the exclusive ERM protein in inner and outer hair cells and the dominant protein in hepatocytes. The concept of radixin-mediated canalicular localization of MRP2 was later confirmed in human cholestatic liver disease patients (94). Recently, this paradigm was extended to apical membrane localization of MRP2 in Caco-2 intestinal cells. Stable knockdown of radixin or ezrin using siRNA resulted in loss of MRP2 from the cell surface, while MRP2 and actin were detected in immunoprecipitates of cell lysates using anti-radixin or anti-ezrin antibodies (95). These results indicate that unlike the situation in hepatocytes, both ezrin and radixin are independently required for proper (apical membrane) localization of MRP2 in Caco-2 intestinal cells. Raft association of MRP2, actin and ERM proteins were not analyzed in these studies (93-95). While ERM protein-mediated linkage to the actin cytoskeleton is beneficial or even essential to the transport

activities of Pgp and MRP2, in the case of the ABC transporter CFTR it was shown that it is held in an immobile state in the membrane by its interaction with actin via ezrin. Moreover, CFTR appears to be indirectly linked to ezrin via the ezrin-binding protein EBP50 (96).

Associations of MRP1 and BCRP with the actin cytoskeleton either direct or via lipid rafts, have not been reported. We have recently obtained evidence for a role of the actin cytoskeleton in lipid rafts localization and function of MRP1 in Neuro-2a and BHK-MRP1 cells. Latrunculin B treatment in these cells results in both reduced association of MRP1 and actin with detergent-free lipid raft fractions and reduced MRP1-mediated efflux function (Klappe K., Hummel I., Mészáros P., Ercan C., Kroesen B.J., Sietsma H., and Kok J.W., unpublished observations).

Concluding remarks

The central question in this review is: Are lipid rafts involved in ABC transporter-mediated drug resistance of tumor cells? (with special reference to Pgp, MRP1 and BCRP). In our view, this question can be tentatively answered positively, although a final conclusion cannot yet be drawn. Substantial evidence has accumulated showing that these three ABC transporters are enriched in DRMs and lipid rafts. In fact, the concept emerges that these transporters may be localized in a specific type of lipid rafts, which is non-caveolar and non-classical. Especially with Pgp, many studies have been conducted to manipulate cholesterol levels in DRMs and lipid rafts, to obtain additional evidence for a functional localization in membrane domains of the transporters. However, it remains problematic to unambiguously attribute effects of cholesterol depletion on ABC transporter localization and function to changes in lipid rafts. Alternative explanations include direct effects of cholesterol itself and indirect effects on membrane fluidity. This is especially difficult in the case of M-β-CD treatment, which has a number of other effects in addition to cholesterol extraction, such as extraction of other lipids and proteins and possibly effects on the cytoskeleton. Thus, there is a need for the use of alternative means to modulate/disrupt lipid rafts. Sphingolipid depletion in our hands has not provided the answer, but this manipulation has been very scarcely used while it should be an integral part of the toolbox

for lipid raft study. In our view, much is to be expected from the emerging interactions of ABC transporters in a lipid raft context with the actin cytoskeleton. Indeed, manipulation of actin may be a good tool to interfere with lipid raft integrity and look for effects on ABC transporter localization and function without changing the cellular lipid composition. Moreover, the intriguing hypothesis that ABC transporter function is regulated by a two axes system, combining lateral interactions with lipids and proteins in the plane of the membrane with transverse interactions with the actin cytoskeleton (and possibly integrins linking to the ECM on the opposite side of the plasma membrane) requires our attention. We anticipate that, in analogy to the recent developments in the field of BCR and TCR signaling, lipid raft – actin cytoskeleton interactions will become an important theme in ABC transporter cell biology. In this respect, three issues appear to be of high interest: 1) Is the actin cytoskeleton a stabilizer of ABC transporter localization or are the interactions between the two of dynamic character? 2) Which actin-binding proteins are involved? It seems a logical step to focus on ERM family proteins, given the already established functions of these actin-binding proteins in Pgp, MRP2 and CFTR linkage to actin. However, many other possible linkers exist and should be investigated as well. 3) In case of indirect linkage of ABC transporters to F-actin, what is the nature of the proteins (or lipids?) which interact with actin and actin-binding proteins on the one hand and ABC transporters on the other hand?

Answers to these questions will definitely increase our knowledge of the regulation of ABC transporter function and MDR and may contribute to novel approaches to counteract MDR in tumor cells.

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Chapter 3

Function of MRP1 is not dependent on cholesterol or cholesterol-stabilized lipid rafts

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Abstract

MRP1 has been localized in cholesterol-enriched lipid rafts, which suggests a role for these lipid rafts and/or cholesterol in MRP1 function. Here we show for the first time that nearly complete oxidation of free cholesterol in the plasma membrane of BHK-MRP1 cells did not affect MRP1 localization in lipid rafts or its efflux function, using CFDA as substrate. Inhibition of cholesterol biosynthesis, using lovastatin (LO) in combination with RO 48-8071, resulted in a shift of Mrp1 out of lipid raft fractions, but did not affect Mrp1-mediated efflux in Neuro-2a cells. Short-term methyl-β-cyclodextrin treatment was equally effective in removing free cholesterol from Neuro-2a and BHK-MRP1 cells, but affected MRP1 function only in the latter. The kinetics of loss of both MRP1/Mrp1 efflux function and lipid raft association during long-term methyl-β-cyclodextrin treatment did not match the kinetics of free cholesterol removal in both cell lines. Moreover, MRP1 activity was measured in vesicles consisting of membranes isolated from BHK-MRP1 cells using the substrate cysteinyl leukotriene C_4 and was not changed when the free cholesterol level of these membranes was either decreased or increased. In conclusion, MRP1/Mrp1 activity is not correlated with the level of free cholesterol or with localization in cholesterol-dependent lipid rafts.

Introduction

One of the best characterized multidrug resistance mechanisms is the energy-dependent drug efflux by proteins belonging to the ABC transporter protein superfamily. MRP1 and Pgp are the most widely studied ABC transporters and are known to depend on their direct lipid environment for optimal functioning (1,2). Lavie et al. (3) have shown for the first time the association of an ABC transporter with a membrane domain. They found that a substantial fraction of Pgp was located in caveolin-1 (Cav-1) containing Triton X-100-based detergent resistant membranes in P-glycoprotein overexpressing cells. Later studies showed localization of both P-glycoprotein and MRP1 in non-caveolar DRMs. Both ABC transporters were more strongly enriched in Lubrol-based or Brij-based DRMs compared to Triton X-100-based DRMs (4,5).

Given their localization in DRMs, the function of ABC transporters may well be dependent on cholesterol, which is known to be enriched in DRMs. Modulation of P-glycoprotein function by cholesterol and involvement of DRMs in this process are widely studied (6). For example, cholesterol depletion resulted in a shift of P-glycoprotein out of DRM fractions and P-glycoprotein-mediated drug transport was also affected (7). In Caco-2 cell monolayers, cholesterol depletion significantly impaired the efflux activity of P-glycoprotein (8). Concerning modulation of MRP1 function by cholesterol, information is scarce. In one study, cholesterol was reported to modulate MRP1 function and this was related to the presence of MRP1 in DRMs (9). It is important to rigorously establish whether cholesterol affects MRP1 function, as this could be the underlying mechanism for the appearance of MRP1 in lipid rafts and a potential target for manipulation of MRP1 activity in the context of tumour cell sensitization to cytostatics.

In this study we rigorously investigated the impact of cholesterol and the localization of MRP1/Mrp1 in cholesterol-dependent lipid rafts on efflux function of the ABC transporter in two cell lines. We used Neuro-2a (neuroblastoma) cells, which express endogenous murine Mrp1 and BHK-MRP1 (fibroblast) cells, which highly express stable transfected human MRP1 (10). In addition, we studied MRP1 function in vesicles consisting of membranes isolated from cholesterol-modulated BHK-MRP1 cells. In view of

potential drawbacks using detergent-based protocols for lipid raft isolation (11,12), we employed the detergent-free lipid raft isolation procedure recently developed by Macdonald and Pike (13). Various strategies were employed to manipulate cellular cholesterol levels, including 1) short- and long-term methyl-β-cyclodextrin treatment, which physically removes cholesterol from the plasma membranes of cells, 2) cholesterol oxidase treatment, which chemically converts cholesterol into cholestenon and 3) combined treatment with lovastatin, an inhibitor of 3-hydroxy-3methylglutaryl-coenzyme A reductase and RO 48-8071, an inhibitor of oxidosqualene cyclase. Mrp1/MRP1 activity did not correlate with the variable levels of cholesterol, either in intact cells or isolated membranes. Neither did Mrp1/MRP1 activity correlate with the protein's variable extent of lipid raft localization resulting from cholesterol modulation. We conclude that Mrp1/MRP1 function is not dependent on cholesterol or cholesterol-dependent lipid raft localization, while the observed effect of (long-term) M-β-CD treatment in intact cells, likely relates to reduced membrane integrity.

Materials and methods

Materials

MK571 was a gift from Prof. A.W. Ford-Hutchinson (Merck-Frosst, Inc., Kirkland, Canada). All cell culture plastic was from Costar (Cambridge, MA, USA). Cell culture media, Hank's balanced salt solution (HBSS), antibiotics, L-glutamine, sodium pyruvate and trypsin were purchased from Gibco (Invitrogen, Paisley, UK). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). RO 48-8071 was from Enzo Life Sciences AG (Farmingdale, NY, USA). Cholesterol oxidase was purchased from Calbiochem (Merck KgaA, Darmstadt, Germany). CFDA, cysteinyl leukotriene C_4 (LTC_4), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), lovastatin, and methyl-β-cyclodextrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]LTC_4 was purchased from PerkinElmer (Waltham, MA, USA). Cholesterol was from Avanti Polar Lipids (Alabaster, AL, USA). The rat monoclonal anti-MRP1 (MRPr1) antibody was

obtained from Alexis (Dedham, MD, USA). GF/C short drop filterplates were from Whatman (Kent, UK). OptiPrep was from Axis-Shield PoC AS (Dundee, Scotland).

Cell culture and incubation conditions

The murine neuroblastoma cell line Neuro-2a was purchased from the ATCC (Manassas, VA, USA). These cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate, under standard incubator conditions (humidified atmosphere, 5% CO_2, 37 °C). The hamster BHK cell line stably expressing the human *MRP1* gene, named BHK-MRP1, was a gift from Dr. Riordan (Mayo Clinic Arizona, S.C. Johnson Medical Research Center, Scottsdale, AZ, USA; ref. 10). These cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium/NUT mix F-12 (1:1) supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, under standard incubator conditions (humidified atmosphere, 5% CO_2, 37 ^{o}C). The cells were kept under selective pressure by growing them in the presence of 100 µM methotrexate. In order to deplete cholesterol, cells were incubated in the presence of 10 mM M-β-CD for various time intervals in serum-free medium. Alternatively, Neuro-2a cells were incubated for 20 h in the presence of both 1 µM RO 48-8071, an inhibitor of oxidosqualene cyclase, and 1 µg/ml lovastatin, an inhibitor of 3-hydroxy-3methylglutaryl-coenzyme A reductase, in serum-free medium. As a control in this case, cells were grown in serum-free medium for 20 h with vehicle. In order to chemically convert cholesterol to cholestenon, cells were incubated in the presence of 1 U/ml cholesterol oxidase for 1 h. For cell membrane preparations cells were grown in roller bottles, harvested by trypsinization, and then subjected to cholesterol oxidase or M-β-CD treatment for various time intervals to deplete plasma membrane cholesterol. For cholesterol loading of the plasma membrane, M-β-CD filled with cholesterol (M-β-CD/cholesterol) was used for different incubation times in serum-free medium at 37 °C (10 mM concentration). To prepare M-β-CD/cholesterol, 100 mg M-β-CD was dissolved in 2 ml water and 3 mg cholesterol (solution in ethanol) was added slowly while stirring at 60-70 °C. This was dried and used later as described above. After treatment cells were

centrifuged (3000 rpm, 3 min), frozen in liquid nitrogen and stored at -80 °C until membrane isolation. Control cells were treated similarly but in the absence of cholesterol modulators, i.e. solvent controlled when applicable.

Isolation of detergent-free lipid rafts

Detergent-free lipid rafts were isolated as described (13,14).

Immunoblot analysis

Protein from equal volumes of the gradient fractions was processed as described (14).

Cholesterol determination

To quantify free cholesterol, a lipid extraction (15) on cells or pooled detergent-free lipid fractions was performed after a protein determination (16). The cholesterol concentration was determined spectrophotometrically by a cholesterol oxidase/peroxidase assay (17). The amount of cholesterol was expressed relative to the protein content.

Sphingolipid analysis by liquid chromatography-electrospray ionization tandem mass spectrometry

Sphingolipids were extracted and analyzed by LC-ESI-MS/MS as described previously (14). The amounts of individual sphingolipid species were added to obtain the total sphingolipid pool. Protein content of pooled OptiPrep gradient fractions was determined as described by Smith et al. (16).

Detection of Mrp1/MRP1-mediated efflux by flow cytometric analysis

Neuro-2a or BHK-MRP1 cells were plated to confluence in 25 cm^2 flasks one day prior to the experiment. Mrp1/MRP1-mediated efflux of the substrate CFDA was performed by flow cytometric analysis as described (18).

Measurement of cellular sensitivity to cytotoxic drugs (MTT assay)

One thousand cells/well were plated in microtiter plates. For depletion of cholesterol, cells were washed 24 h after plating with serum-free medium and incubated in the presence of 10 mM M-β-CD in serum-free medium for 1h at 37 °C. Subsequently, cells were washed with serum-free medium and incubated for 48h in serum-containing medium. Viable cells were determined 72h after plating as previously described (14).

Isolation of membrane vesicles from BHK-MRP1 cells

BHK-MRP1 cells were washed with HBSS, trypsinized, harvested and treated, then centrifuged (3000 rpm, 3 min), frozen in liquid nitrogen and stored in -80 °C. Pellets of ~ $8x10^8$ frozen cells were resuspended in hypotonic solution (5 mM sodium phosphate, pH 7.4, containing protease inhibitors and 1 mM EDTA) by stirring for 90 min on ice. After centrifugation (100000 x g, 45 min, 4 °C) cell material was resuspended in isotonic buffer (10 mM Tris, pH 7.4, 250 mM sucrose, containing protease inhibitors and 1 mM EDTA) and homogenized using a Dounce tissue grinder. The material was carefully layered on top of a 38% sucrose solution and centrifuged (280000 x g, 2 h, 4 °C). The membrane interface was collected and diluted in isotonic buffer and centrifuged again (100000 x g, 45 min, 4 °C). The membrane pellet was finally resuspended in isotonic buffer at a concentration of 5 mg/ml of protein (16), frozen in liquid nitrogen and stored at -80 °C.

Measurement of MRP1-mediated ATPase and transport activities

Vanadate sensitive ATPase activites were measured as described earlier (19,20). Briefly, isolated membranes (8 µg/well) were incubated for 11 min in 50 µl ATPase assay mix (40 mM MOPS-Tris pH 7.0, containing 10 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 0.1 mM glycol ether diamine tetraacetic acid (EGTA), 4 mM Na-azide, and 5 mM ATP). The reaction was stopped with 100 µl malachite green mix (21-25), as modified in our lab (5.6 mM malachite green, 9.4 mM ammonium-molybdate, 2 % citric acid, 4.6 % ethanol, and 1.16 N HCl, final concentration). After two min 100 µl of citric acid was added and incubated at 37 °C for 15-20 min. The absorbance values were measured using a spectrophotometer (Biotek uQuant) at a wavelength of 630 nm. For transport of LTC_4 into

membrane vesicles, vesicles were incubated as described before (26) in 40 mM MOPS-Tris pH 7.0, 180 mM sucrose, containing 10 mM $MgCl_2$, 20 mM KCl, 47.8 nM LTC_4 and with or without 4 mM ATP, for various time intervals at 37 °C. LTC_4 at a concentration of 47 nM was mixed with [^{3}H]LTC_4 (specific activity of 190 Ci/mmol) at a concentration of 0.8 nM. The reaction was stopped with ice cold washing buffer (10 mM Tris pH 7.0, 180 mM sucrose, 20 mM KCl). The reaction mix was rapidly filtered in glass fibre filter plates (Whatman GF/C short drop), washed with 5x200 µl ice cold washing buffer, and dried. Radioactivity was measured by liquid scintillation counting (Packard Topcount microplate scintillation counter). ATPase activity was expressed as pmol Pi/mg/min and transport into membrane vesicles as pmol LTC_4/mg/min. For graphic representation, values were normalized to maximal activity in control conditions (=100%).

Measurement inside-out vesicle content

Determination of the inside-out vesicle ratio was based on 5'-nucleotidase activity and performed as described earlier (27) with the following changes: 25-50 µg of membrane vesicles was incubated (30 min, 37 °C) in 50 mM Tris, pH 7.4, containing 4 mM $MgCl_2$, and with or without 3 mM AMP and/or 0.3 % Triton X-100. The inorganic phosphate released by the 5'-nucleotidase was measured with the malachite green system as described above. 5'-nucleotidase activity was measured in 4 conditions; A: with AMP and Triton X-100, B: with AMP, without Triton X-100, C: without AMP, with Triton X-100 and D: without AMP, without Triton X-100. A-C yields the total activity of the enzyme, B-D the activity of the right-side-out vesicles only. The percentage of inside-out vesicles was calculated as [[(A-C)-(B-D)]/(A-C)]x100%.

Results

Efficient depletion or uploading of cholesterol

First we established the efficacy of cholesterol modulation, using three different strategies. 1) M-β-CD was used to physically deplete cholesterol from cell membranes. 2) Lovastatin, an inhibitor of 3-hydroxy-3methylglutaryl-coenzyme A reductase, plus RO 48-8071, an

inhibitor of oxidosqualene cyclase, were used to inhibit the biosynthesis of cholesterol. 3) Cholesterol oxidase was used to chemically convert cholesterol to cholestenon.

The results are summarized in Table 1. In Neuro-2a cells a 1 h treatment with 10 mM M-β-CD decreased the cholesterol level by 71.6% in whole cells. In detergent free lipid raft fractions from these cells, cholesterol was even more strongly depleted (87.0%). In BHK-MRP1 cells cholesterol depletion by M-β-CD was 64.1%. The M-β-CD treatment slightly affected Neuro-2a cell viability, as determined by a MTT assay. Neuro-2a cell viability was 81.4% ± 18.6% (n=6) for M-β-CD-treated cells (1 h) compared to control cells (=100%).

Cell type	Fraction	Method	Cholesterol content (control = 100%)
Neuro-2a	whole cells	M-β-CD	28.4% ± 12.3
Neuro-2a	detergent-free lipid rafts	M-β-CD	13.0% ± 7.5
Neuro-2a	whole cells	lovastatin + RO 48-8071	47.1% ± 7.8
Neuro-2a	whole cells	cholesterol oxidase	71.0% ± 5.0
BHK-MRP1	whole cells	M-β-CD	35.9% ± 3.0
BHK-MRP1	whole cells	cholesterol oxidase	3.7% ± 2.1

Table 1. Cholesterol depeletion methods and efficiency in intact cells
Various procedures were performed to manipulate cholesterol levels, including M-β-CD treatment (10 mM, 1 h), which physically removes cholesterol from the plasma membranes of cells, cholesterol oxidase treatment (1 U/ml, 1 h), which chemically converts cholesterol into cholestenon and combined treatment with lovastatin (1 µg/ml), and RO 48-8071 (1 µM), an inhibitor of oxidosqualene cyclase. Cholesterol content was measured and normalized to protein. These values were normalized to control (=100%). Data indicate the mean ± SD of three independent measurements.

Conditions for inhibition of cholesterol biosynthesis in Neuro-2a cells were optimized in terms of concentrations and time of incubation (data not shown). Cells incubated for 20 h in the presence of both 1 µg/ml lovastatin and 1 µM RO 48-8071 showed a 52.9% decrease of the cholesterol level. Finally, when 1 U/ml cholesterol oxidase was used for 1 h in Neuro-2a cells, the cholesterol content was reduced by 29.0%. In BHK-MRP1 cells, the enzyme was much more efficient, reducing cholesterol by 96.3%.

The cholesterol levels in cell membranes isolated from cholesterol-modulated BHK-MRP1 cells are summarized in Table 2.

Cholesterol depletion with Cholesterol Oxidase (CO)		Cholesterol depletion with M-β-Cyclodextrin (CD)		Cholesterol upload with M-β-CD/cholesterol (CDC)	
Treatment	Cholesterol (%)	Treatment	Cholesterol (%)	Treatment	Cholesterol (%)
COa	100	CDa	100	CDCa	100
COb	75 ± 1,8	CDb	71 ± 4,8	CDCb	136 ± 15,2
COc	63 ± 6,8	CDc	53 ± 2,6	CDCc	143 ± 17,7
COd	52 ± 1,2	CDd	43 ± 5.1	CDCd	158 ± 23,3

Table 2. Cholesterol modulation methods and efficiency in isolated membranes from BHK-MRP1 cells
BHK-MRP1 cells were treated during various time intervals with cholesterol oxidase (CO; 1 U/ml), M-β-CD (CD; 10 mM), or M-β-CD/cholesterol (CDC; 10 mM). Each treatment was performed independently three times, resulting in three independent membrane preparations. Cholesterol content was measured and normalized to protein. These values were normalized to control (=100%). Data indicate the mean ± SD of three independent membrane preparations. (a) represents membranes from control cells and (b), (c) and (d) membranes from cells treated for various time intervals with CO, CD or CDC, respectively. In case of CO, (b), (c) and (d) refer to 30, 60 and 90 min treatment, respectively. In case of CD and CDC, this is 10, 20 and 30 min.

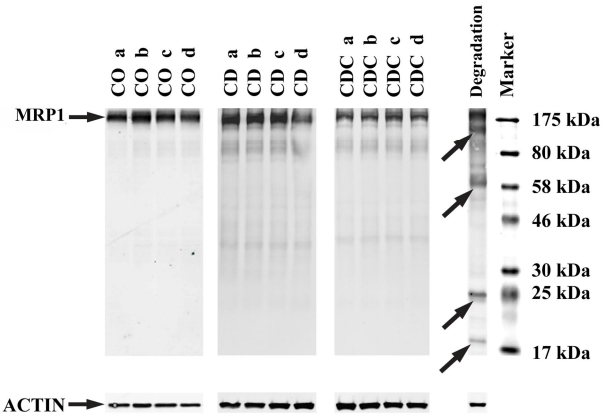

Figure 1. MRP1 integrity in membranes isolated from BHK-MRP1 cells
BHK-MRP1 cells were treated with cholesterol oxidase (CO; 1 U/ml), M-β-CD (CD; 10 mM), or M-β-CD/cholesterol (CDC; 10 mM). Cell membranes were isolated from these cells and control cells. For each treatment, three independent membrane preparations were generated. Typical results out of three independent experiments for each treatment are shown. (a) represents membranes from control cells and (b), (c) and (d) membranes from cells treated for various time intervals with CO, CD or CDC, respectively. In case of CO, (b), (c) and (d) refer to 30, 60 and 90 min treatment, respectively. In case of CD and CDC, this is 10, 20 and 30 min. Membranes were loaded on SDS-PAGE using 10 µg of protein/sample. Western blots were stained for MRP1 and actin. "Degradation" refers to the sample in which cholesterol oxidase treatment (30 min, 37 ºC) was performed on membranes after they had been isolated from cells, which resulted in partial degradation of MRP1 (arrows indicate bands at ~150, ~60, ~25 and ~20 kDa). This suggests that the treatments performed on the cells (b,c,d) did not result in MRP1 degradation, as compared to control conditions (a).

Cholesterol oxidase treatment of intact BHK-MRP1 cells resulted in a maximal decrease in cholesterol level of ~50% in the isolated cell membranes. This was less efficient compared to previous experiments (Table 1), possibly due to the use of large scale cell cultures needed for the subsequent isolation of membranes. We also treated isolated membrane vesicles with cholesterol oxidase, but this resulted in degradation of MRP1 (Fig. 1) and these preparations were therefore not suitable for analysis of MRP1 function. MRP1 was not degraded in membrane preparations obtained from cholesterol-modulated BHK-MRP1 cells, as compared to control membrane preparations (Fig. 1).

Cholesterol depletion by M-β-CD treatment reduced cholesterol by ~57% in isolated membranes, while cholesterol uploading with M-β-CD/cholesterol increased the cholesterol level by ~58%. The cholesterol content of control membrane vesicles was 240 ± 30 nmol cholesterol/mg protein (n=9).

Cholesterol does not affect efflux function of Mrp1/MRP1

Having established the extent of cholesterol modulation with three different strategies, we next measured the effects on Mrp1/MRP1-mediated CFDA efflux. Efflux activity was the same in cholesterol oxidase-treated and control cells, regarding both Neuro-2a (Fig. 2A) and BHK-MRP1 (Fig. 2B) cells. Also in lovastatin/RO 48-8071-treated Neuro-2a cells, efflux activity was similar to control (Fig. 2C). MK571 was used as a positive control for inhibition of Mrp1/MRP1-mediated efflux (Fig. 2A-C). With M-β-CD differential effects on Mrp1/MRP1-mediated efflux kinetics were observed in the two cell types. In Neuro-2a cells, efflux activity was normal up to 10 min of M-β-CD treatment, but was reduced after 30 min and maximally affected after 45 min treatment (Fig. 2A). In BHK-MRP1 cells, on the other hand, MRP1-mediated efflux activity was already reduced after 10 min of M-β-CD treatment, and subsequently kept fluctuating around this level (Fig. 2B). It was therefore important to measure in parallel the kinetics of cholesterol depletion in both cell types, which turned out to be very similar in Neuro-2a and BHK-MRP1 cells (Fig. 4). Cholesterol levels were equally decreased at t = 10 min in both cell types (Fig 4.)

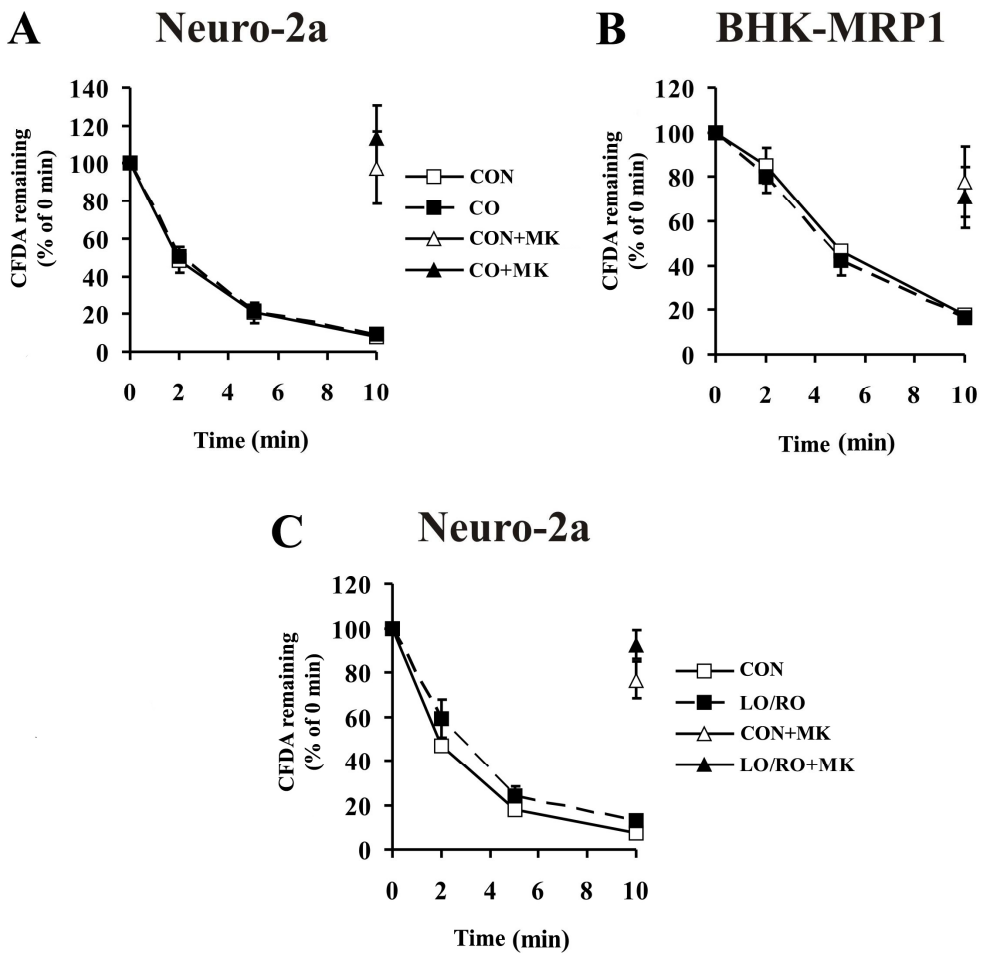

Figure 2. Cholesterol modulation does not affect Mrp1/MRP1-mediated efflux in Neuro-2a and BHK-MRP1 cells

A,B: Neuro-2a (A) or BHK-MRP1 cells (B) were untreated (CON) or treated with 1 U/ml cholesterol oxidase for 1 h (CO). **C:** Neuro-2a cells were untreated (control: 20 h in serum-free medium) or treated with 1 µg/ml lovastatin + 1 µM RO 48-8071 for 20 h in serum-free medium (LO/RO). Subsequently, cells were loaded with CFDA (0.5 µM) and allowed to efflux at 37°C during several time intervals. The remaining cell-associated fluorescence was determined by cytometric analysis and expressed as % of the 0 min value. Control: open squares; CO or LO/RO: closed squares. MK571 (20 µM) was used as a positive control for Mrp1/MRP1 efflux inhibition. Control + MK571: open triangles; CO + MK571 or LO/RO + MK571: closed triangles. Data represent the mean ± SD (n=3).

Interestingly, upon longer M-β-CD treatment, both cell types became sensitive to MK571, as indicated by loss of Mrp1/MRP1 substrate likely due to membrane leakage. This occurred between 45 and 60 min for Neuro-2a (Fig. 3C) and between 15 and 30 min for BHK-MRP1 cells (Fig. 3D).

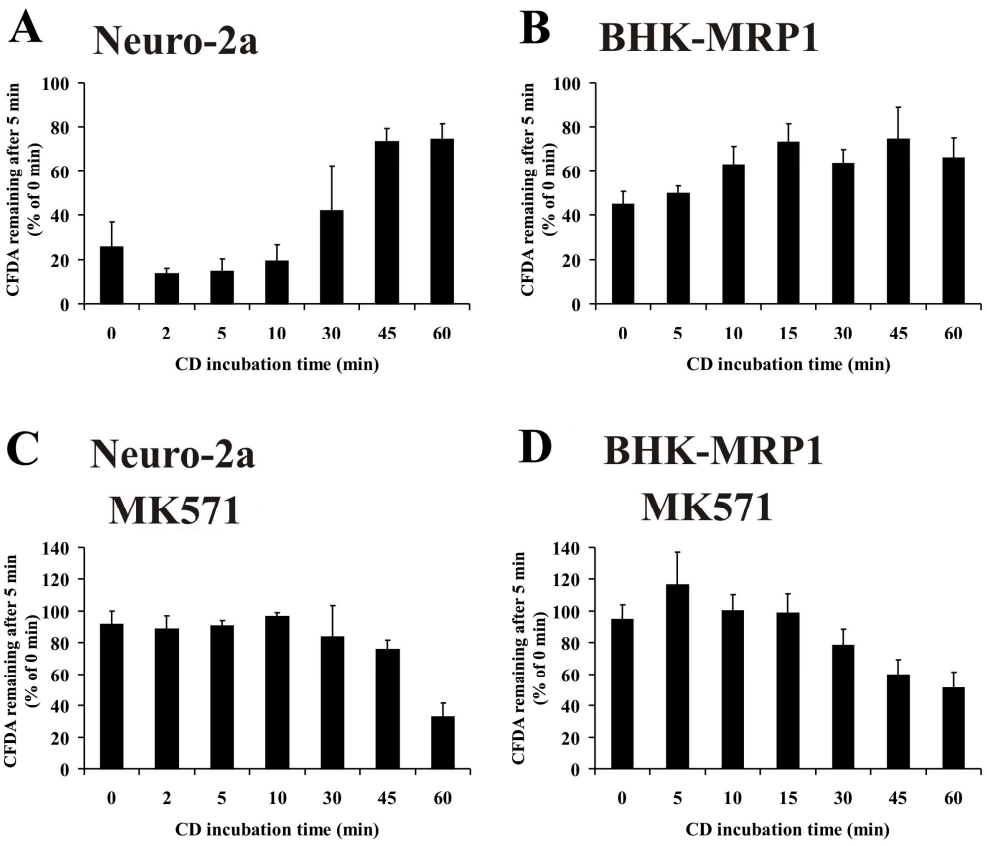

Figure 3. Effects of M-β-CD treatment on Mrp1/MRP1-mediated efflux in Neuro-2a and BHK-MRP1 cells

Neuro-2a (A,C) or BHK-MRP1 cells (B,D) were treated with 10 mM M-β-CD for various time intervals. Subsequently, cells were loaded with CFDA (0.5 μM) and allowed to efflux at 37 °C for 5 min. The remaining cell-associated fluorescence was determined by cytometric analysis and expressed as % of the 0 min value. MK571 (20 μM) was used as a control for Mrp1/MRP1 efflux inhibition (C,D). Data represent the mean + SD (n=3).

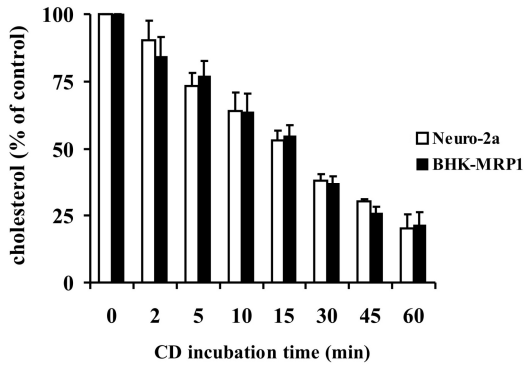

Figure 4. Kinetics of cholesterol depletion upon M-β-CD treatment in Neuro-2a and BHK-MRP1 cells
Neuro-2a (open bars) or BHK-MRP1 cells (closed bars) were treated with 10 mM M-β-CD for various time intervals. Subsequently, cholesterol content of the cells was determined and normalized to protein content. Values are expressed as the percentage of cholesterol in untreated cells (=100%). Data represent the mean + SD (n=3).

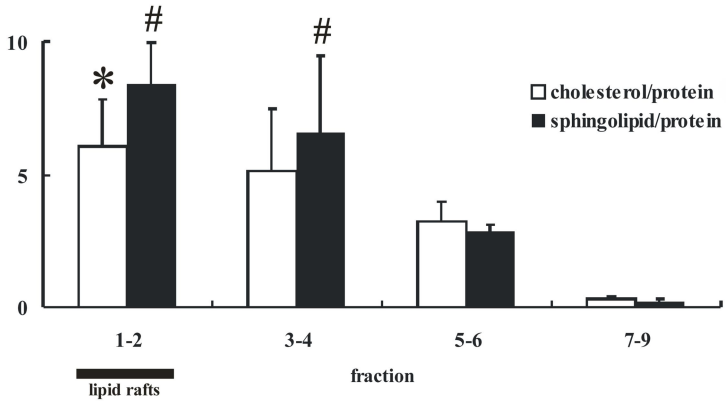

Figure 5. Enrichment of cholesterol and sphingolipid relative to protein in detergent-free lipid raft gradients of Neuro-2a cells
Detergent-free lipid raft gradients were analyzed for cholesterol (open bars) and sphingolipid (closed bars) profile. These lipid classes were measured in pooled gradient fractions and are expressed relative to protein content of the pooled gradient fractions. To calculate the ratio cholesterol/protein and sphingolipid/protein, the values of % in the gradient of each, cholesterol, sphingolipids and protein, were used. This ratio is therefore without unit and represents the relative enrichment in gradient fractions of cholesterol and sphingolipids, respectively, relative to protein enrichment in the respective fractions. Data represent the mean + SD of 3 independent experiments. *Values are significantly ($P < 0.05$) different from the cholesterol/protein ratio of fractions 5-6 (non-lipid raft membrane fractions) as determined by Student's *t*-test. #Values are significantly ($P < 0.05$) different from the sphingolipid/protein ratio of fractions 5-6 (non-lipid raft membrane fractions) as determined by Student's *t*-test.

Mrp1/MRP1 efflux function is not correlated with its localization in lipid rafts

We used a detergent-free method for the isolation of lipid rafts and first characterized the gradient fractions in terms of cholesterol and sphingolipid enrichment. For this purpose, fractions 1-2 were pooled, as well as 3-4, 5-6 and 7-9. Fractions 1-2 were most strongly enriched in both cholesterol and sphingolipids (Fig. 5), and to a lesser extent also fractions 3-4. This indicates that fractions 1-2, with the lowest buoyant density, optimally fulfil the criteria for lipid rafts. In accordance with the absence of an effect of cholesterol oxidation on Mrp1 efflux function, there was no effect on detergent-free lipid raft localization of the ABC transporter, as indicated by a similar gradient profile of Mrp1 compared to control Neuro-2a cells (Fig. 6A). Cholesterol oxidase was much more effective in reducing cholesterol content in BHK-MRP1 cells than in Neuro-2a cells. Therefore, we also rigorously tested lipid raft association of MRP1 under cholesterol oxidase conditions in BHK-MRP1 cells and this turned out to be equal to control cells, as confirmed by quantification of the lipid raft-associated pools (Fig. 6B).

On the other hand, lovastatin/RO 48-8071 treatment did result in a clear shift of Mrp1 out of detergent-free lipid raft fractions in Neuro-2a cells, as confirmed by quantification of the relative amount of Mrp1 in lipid raft fractions (Fig. 6C). Upon M-β-CD treatment, detergent-free lipid raft association of Mrp1 was gradually reduced in Neuro-2a cells and this was significant after 60 min (Fig. 7A). In BHK-MRP1 cells, MRP1 showed a tendency to shift out of lipid raft gradient fractions only after 60 min M-β-CD treatment, but this did not reach significance due to large variation (Fig. 7B).

MRP1 ATPase and transport activities are not correlated with cholesterol levels in isolated cell membranes

The absence of effects of cholesterol modulation on Mrp1/MRP1 function could have been due to confounding factors in the complex background of the intact cell. To overcome the complexity of the cell system, plasma membrane vesicles were isolated from cholesterol modulated BHK-MRP1 cells. To make sure that our procedures to modulate cholesterol in cells did not affect the inside-out ratio of MRP1 in the isolated membrane vesicles, we

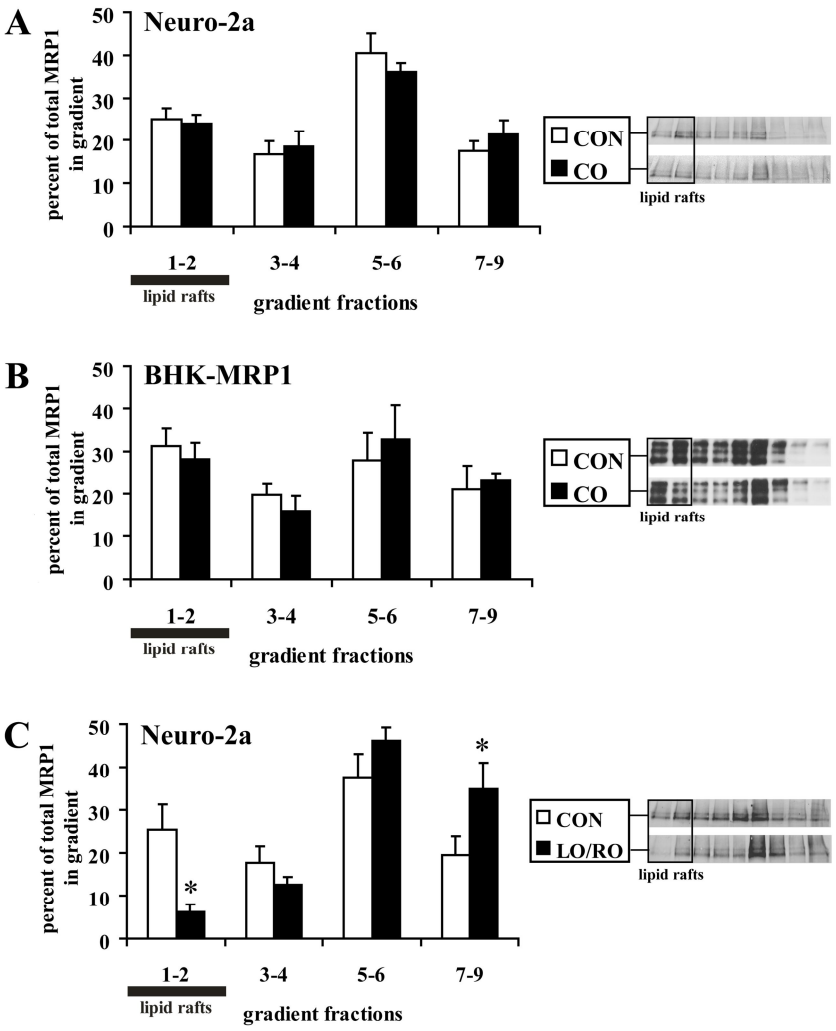

Figure 6. Detergent-free lipid raft localization of Mrp1 is affected by lovastatin/RO 48-8071 but not by cholesterol oxidase in Neuro-2a cells (and BHK-MRP1 cells)
Cells were untreated (CON) or treated with either 1 U/ml cholesterol oxidase for 1 h (CO) or with 1 µg/ml lovastatin + 1 µM RO 48-8071 for 20 h in serum-free medium (LO/RO). A: Lipid raft association of Mrp1 in CO-treated Neuro-2a cells compared to control. B: Lipid raft association of MRP1 in CO-treated BHK-MRP1 cells compared to control. C: Lipid raft association of Mrp1 in LO/RO-treated Neuro-2a cells compared to control. The numbers indicate the percentage of Mrp1/MRP1 found in the pooled gradient fractions, relative to total Mrp1/MRP1 in the entire gradient (9 fractions). Data represent the mean + SD (n=3). Asterisks indicate values that are significantly different from CON ($P < 0.05$, as determined by Student's *t*-test). The right panels show representative blots from three experiments concerning Mrp1/MRP1 distribution along the gradient. Gradient samples were applied on SDS-PAGE based on equal volume. Lipid raft fractions are indicated.

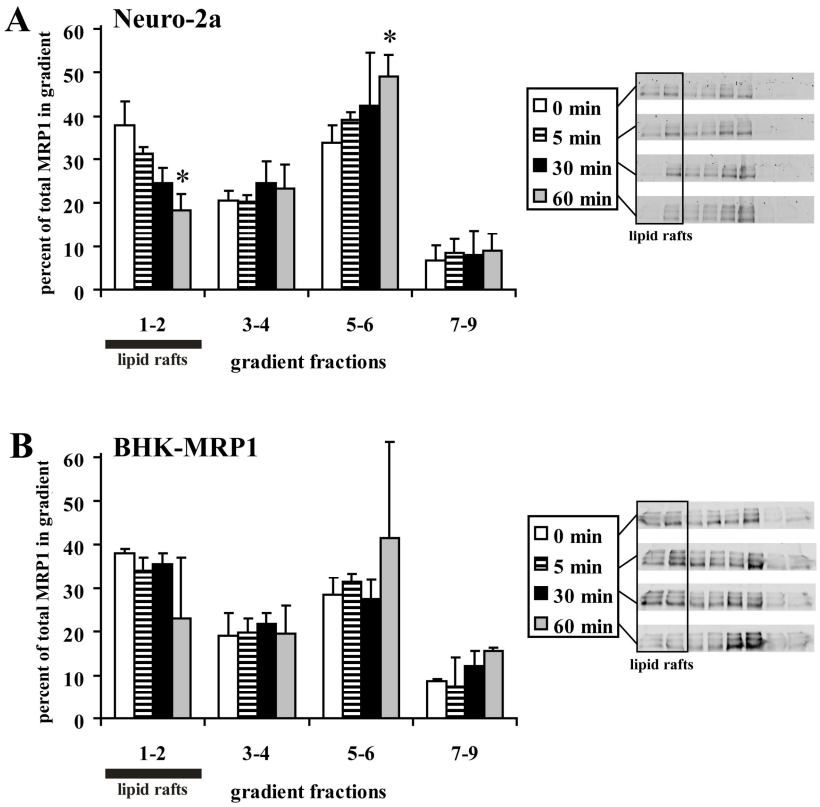

Figure 7. Effects of M-β-CD treatment on detergent-free lipid raft localization of Mrp1/MRP1 in Neuro-2a and BHK-MRP1 cells
Neuro-2a (**A**) or BHK-MRP1 cells (**B**) were untreated (0 min) or treated with 10 mM M-β-CD for various time intervals (5, 30, 60 min) and lipid raft association of Mrp1/MRP1 was determined under these conditions. The numbers indicate the percentage of Mrp1/MRP1 found in the gradient fractions, relative to total Mrp1/MRP1 in the entire gradient (9 fractions). Data represent the mean + SD (n=3). Asterisks indicate values that are significantly different from CON ($P < 0.05$, as determined by Student's *t*-test). The right panels show representative blots from three experiments concerning Mrp1/MRP1 distribution along the gradient. Gradient samples were applied on SDS-PAGE based on equal volume. Lipid raft fractions are indicated.

measured this ratio. Under all conditions, the inside-out vesicle ratio was found to be invariable (Fig. 8). Moreover, MRP1 integrity was not changed by our cholesterol modulation procedures, as shown by Western blot (Fig. 1).

In this simplified system for MRP1 function analysis, we measured ATPase activity of MRP1 as well as transport of LTC_4 into membrane vesicles by MRP1 (Fig. 9). The

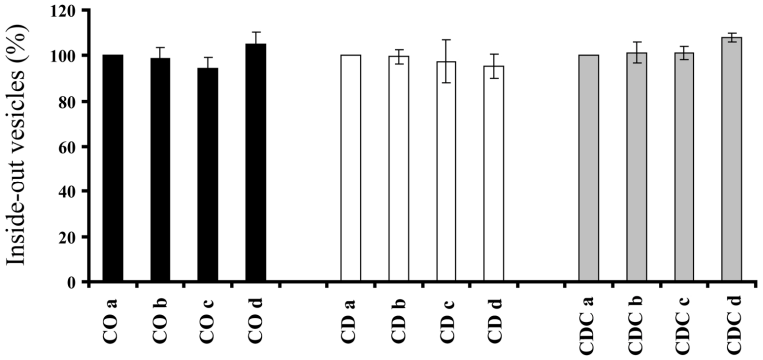

Figure 8. Different cholesterol content does not affect the inside-out ratio of the membrane vesicles.
BHK-MRP1 cells were treated with cholesterol oxidase (CO; 1 U/ml), M-β-CD (CD; 10 mM), or M-β-CD/cholesterol (CDC; 10 mM). Cell membranes were isolated from these cells and control cells. For each treatment, three independent membrane preparations were generated. For each membrane preparation, the measurements were performed in triplicate. (a) represents membranes from control cells and (b), (c) and (d) membranes from cells treated for various time intervals with CO, CD or CDC, respectively. In case of CO, (b), (c) and (d) refer to 30, 60 and 90 min treatment, respectively. In case of CD and CDC, this is 10, 20 and 30 min. The inside-out ratio of all membrane preparations was determined using the 5'-nucleotidase assay. All ratios were normalized to control (a=100%). The inside-out ratio of control membranes varies between 20-30%. The values represent mean ± SD of three independent membrane preparations. There were no significant differences between control (a) and the corresponding (b), (c) and (d) samples (Student's *t*-test).

Treatment	Kruskal-Wallis test	
	ATPase (P value)	LTC_4 transport (P value)
Cholesterol depletion with Cholesterol Oxidase (CO)	0.147	0.168
Cholesterol depletion with M-β-CD (CD)	0.121	0.192
Cholesterol uploading with M-β-CD/cholesterol (CDC)	0.622	0.063

Table 3. Statistical analysis of vanadate sensitive ATPase activity and transport of LTC_4 into membrane vesicles by MRP1 in cholesterol modified membrane vesicles from BHK-MRP1 cells
BHK-MRP1 cells were treated with cholesterol oxidase (CO; 1 U/ml), M-β-CD (CD; 10 mM), or M-β-CD/cholesterol (CDC; 10 mM). Cell membranes were isolated from these cells and control cells. The vanadate sensitive ATPase activity or the transport of LTC_4 into membrane vesicles by MRP1 were measured, as shown in Fig. 10. Per treatment, a Kruskal-Wallis test was performed to analyze statistical differences between the groups (a) to (d), using the area under the curves (see Fig. 5) constructed from three independent membrane preparations. There were no statistical differences (the significance level was set at $p<0.05$).

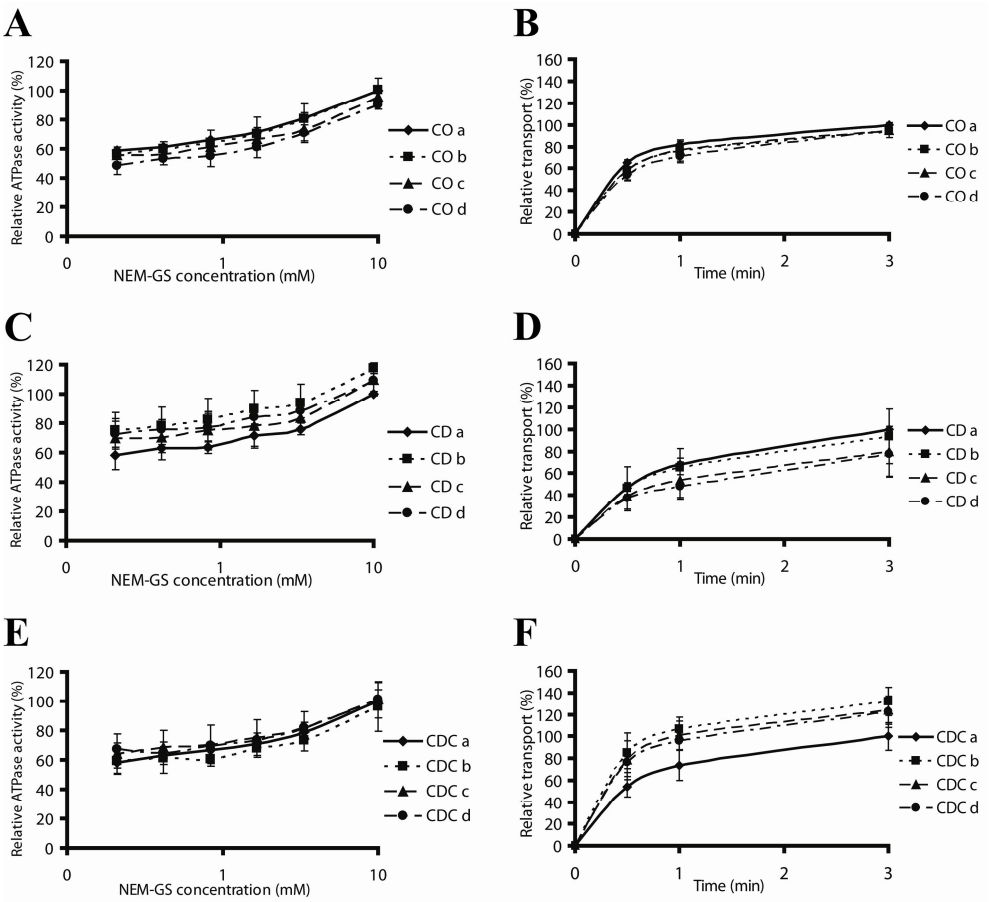

Figure 9. Vanadate sensitive ATPase activity and transport of LTC_4 into membrane vesicles by MRP1 in cholesterol modified membrane vesicles

BHK-MRP1 cells were treated with cholesterol oxidase (CO; 1 U/ml; A,B), M-β-CD (CD; 10 mM; C,D), or M-β-CD/cholesterol (CDC; 10 mM; E,F). Cell membranes were isolated from these cells and control cells. The vanadate sensitive ATPase activity (**A,C,E**) or the transport of LTC_4 into membrane vesicles (**B,D,F**) by MRP1 were measured. For each treatment, three independent membrane preparations were generated. For each membrane preparation, the measurements were performed in triplicate. Each data point in a curve represents the average ± SD of three independent membrane preparations. (a) represents membranes from control cells and (b), (c) and (d) membranes from cells treated for various time intervals with CO, CD or CDC, respectively. In case of CO, (b), (c) and (d) refer to 30, 60 and 90 min treatment, respectively. In case of CD and CDC, this is 10, 20 and 30 min. (a) ♦ solid line; (b) ■ dotted line; (c)▲dashed line; (d) ● dashed dotted line. ATPase activity was expressed as pmol Pi/mg/min and LTC_4 transport as pmol LTC_4/mg/min. For graphic representation, values were normalized to maximal activity in control conditions (=100%).

average vanadate sensitive ATPase activity and transport of LTC_4 into isolated control plasma membranes were 34 ±9 pmol Pi/mg/min (n=27) and 74 ±39 pmol/mg/min (n=27), respectively. There were no significant differences between cholesterol depleted (cholesterol oxidase or M-β-CD treated) or cholesterol uploaded (M-β-CD/cholesterol treated) membranes on the one hand and control membranes on the other (Fig. 9 and Table 3). The average V_{max} and K_m values of the transport of LTC_4 into membrane vesicles were 661 ±171 pmol/mg/min and 942 ±220 nM, respectively. These kinetic parameters of the LTC_4 transport did not show any statistical differences between cholesterol-modulated and control membranes (data not shown).

Discussion

With regard to cholesterol-modulated ABC transporter activity, most studies have focused on P-glycoprotein. These studies have generated much information, but unfortunately not a coherent picture of whether and how cholesterol affects P-glycoprotein function (6,28). Concerning modulation of Mrp1/MRP1 by cholesterol, very little information is available. One study shows that when the cellular cholesterol level is lowered, MRP1 function is reduced and concomitantly MRP1 shifts out of lipid rafts in GLC4 multidrug-resistant lung cancer cells (9). This conclusion was based on results obtained solely with M-β-CD treatment. In our study, we obtained similar effects of M-β-CD treatment. However, we did not attribute these effects to cholesterol, in view of the results obtained with the additional procedures for cholesterol modulation, i.e. cholesterol oxidase and lovastatin plus RO 48-8071. Mrp1/MRP1-mediated efflux was not affected in these two treatment protocols, while the cholesterol level was strongly decreased, especially in cholesterol oxidase-treated BHK-MRP1 cells. Moreover, with M-β-CD treatment efflux was dissociated from the cholesterol level, since short-term treatment did affect efflux in BHK-MRP1, but not in Neuro-2a cells, while cholesterol levels were equally decreased in the two cell types. Long-term M-β-CD treatment in Neuro-2a cells decreased both cholesterol levels and Mrp1-mediated efflux. However, long-term M-β-CD treatment has been shown to result in side effects, such as removal of other lipids and potentially even proteins from membranes

(8,29). Thus, interpretation of such studies should be done with care. The data obtained with MK571 as inhibitor of Mrp1/MRP1 efflux show that after short-term M-β-CD treatment the residual cell associated substrate is high, as expected. However, after long-term M-β-CD treatment substrate is lost from MK571-treated cells, suggesting that MK571 becomes toxic in combination with M-β-CD. This would indicate that membranes gradually destabilize with M-β-CD treatment. It is noteworthy that the M-β-CD effect becomes apparent at shorter incubation times in BHK-MRP1 cells, as compared to Neuro-2a cells. This could be related to the fact that BHK-MRP1 cells were subjected to forced expression of human MRP1, possibly resulting in less stable integration of MRP1 in the plasma membrane. As a consequence, MRP1 would become more prone to the destabilizing effects of M-β-CD. On the other hand, when the M-β-CD effect on Neuro-2a cells sets in, it is more pronounced compared to that in BHK-MRP1 cells.

The studies on intact cells were extended to isolated membrane vesicles to overcome potential confounding effects in the complex system of the intact cell. The modulation of cholesterol performed on the cells did not change the integrity and inside-out ratio of MRP1 in the vesicles consisting of isolated membranes from these cells, allowing us to properly measure potential effects on MRP1 function. Data on MRP1 function in these vesicles with decreased cholesterol levels confirm the conclusion that MRP1 function does not depend on cholesterol. In addition, uploading of cholesterol was ineffective with regard to the function of MRP1. Moreover, this shows that our results are not specific for the Mrp1/MRP1 substrate CFDA, since in this assay for MRP1 function in vesicles from isolated membranes a completely different substrate (LTC_4) was used.

Next we explored whether Mrp1/MRP1-mediated efflux activity was related to localization of Mrp1/MRP1 in cholesterol-stabilized lipid rafts. Comparison of results revealed that all 4 combinations of “effect/no effect” on these two parameters occurred. 1) After cholesterol oxidase treatment, both Mrp1/MRP1-mediated efflux and its localization in lipid rafts were unaffected. 2) Upon long-term M-β-CD treatment both efflux and lipid raft localization of Mrp1/MRP1 were reduced. 3) On the other hand, with short-term M-β-CD treatment in BHK-MRP1 cells, efflux was affected, while raft localization was normal. 4) The opposite occurred after lovastatin plus RO 48-8071-treatment, which resulted in a

shift of Mrp1 out of lipid raft fractions without any effect on efflux activity. Given the fact that all possible combinations of “effect/no effect” on these 2 parameters occurred, we can conclude that Mrp1/MRP1-mediated efflux activity and its localization in cholesterol-stabilized lipid rafts were not correlated. In a recent study we showed that Mrp1/MRP1 efflux function was correlated with its localization in cortical actin-stabilized lipid rafts (18). Therefore, we should not only consider that Mrp1/MRP1 (and other ABC transporters as well) may behave differently between various cell types in terms of (lipid raft) localization-function coupling. In addition, it may well be that within a certain cell type, different types of lipid rafts exist, which are sensitive to either cholesterol or cortical actin, while only the latter is relevant for MRP1 function.

Taken together, we thoroughly investigated potential effects of cholesterol on Mrp1/MRP1 function in two different cell lines and in vesicles from isolated membranes. Moreover, in order not to rely completely on M-β-CD treatment we used 3 different cholesterol modulation procedures. Based in this comprehensive approach, we show for the first time that Mrp1/MRP1 function cannot be categorically linked to cholesterol levels or cholesterol-stabilized lipid raft localization in two different cell lines. A previous study did show an effect on MRP1 function and localization by lowering the level of cholesterol in yet another cell line (9). Therefore, studies in additional Mrp1/MRP1 (over)expressing cell types have to be performed before more general conclusion can be drawn. In any case, cholesterol is not essential for MRP1 function and therefore does not seem to take part in the mechanism underlying functional association of MRP1 with lipid rafts, as has been observed for cortical actin (18).

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Chapter 4

Long term myriocin treatment increases MRP1 transport activity

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Submitted

Abstract

Multidrug resistance-related protein 1 is an important member of the ABC protein super family, which conveys chemoresistance to tumor cells. For therapeutic purposes, it is therefore important to understand the mechanism of action of this transporter protein. Here, we investigated the effect of long term myriocin treatment, which extensively depletes sphingolipids from cells, on MRP1 efflux activity as determined in MRP1 expressing cells and in isolated plasma membrane vesicles. Our data reveal that both short term (3 days) and long term (7 days) treatment with myriocin effectively reduce the cellular sphingolipid levels to the same extent. Intriguingly, this diminishment was without effect on MRP1 activity following short term treatment, but an approximately two-fold increase in efflux activity was observed following long term treatment. Very similar data were obtained by employing plasma membrane vesicles, isolated from myriocin-treated cells. Although subtle changes in phospholipid species were detected following prolonged myriocin treatment, exogenous addition of these species inhibited, rather than promoted MRP1 activity. This suggests that MRP1 activity is not subject to allosteric effects of such lipids. Indeed, exploiting the cell-free vesicle system, Michaelis-Menten analysis revealed that the intrinsic MRP1 activity remained unaltered, but rather the fraction of active transporter molecules increased. We demonstrate that the latter is due to an enhanced recruitment of MRP1 into lipid raft fractions, which thus promotes MRP1 activity.

Introduction

ABC transporters were discovered because of their ability to render cancer cells multidrug resistant. Often cancer cells over express these proteins serving the purpose of defense against chemotherapeutic agents. 49 ABC transporter coding genes were found in the human genome and this super family of membrane proteins has been subdivided into 9 subfamilies. They can transport (efflux) substrates, regulate ion channels or regulate multiprotein channel-complexes (receptors). Because ABC proteins are expressed in many pharmacological barriers and since they can alter pharmacological properties of drugs, nutrients or other molecules, there is a great need for in vitro assays which can predict the interaction between (potential new) drugs and ABC transporters in the drug development process (1). MRP1 is a member of the C subfamily (ABCC1) of the ABC superfamily, and recognizes conjugated organic anions as substrates, such as LTC4 or estrone beta-glucoronide and estrone-sulphate. In most cases transported substrates are glutathione (GSH)-conjugated and the transport utilizes energy from ATP hydrolysis.

There are several factors which can influence the activity of ABC transporters. The precise localization of these proteins in connection to other proteins, such as actin as part of the cytoskeleton may play a role (2). Of importance is the lipid environment, especially when the transporter is localized in lipid rafts. These membrane domains are enriched in sphingolipids and cholesterol and vary in size from 20 to 200 nm. MRP1 is partially located in lipid rafts as shown by several groups (3). This raises the possibility that major components of lipid rafts, i.e. cholesterol and sphingolipids, affect the function of MRP1. Concerning cholesterol, different conclusions have been reached, ranging from a positive effect on MRP1-mediated efflux (4) to the absence of a correlation between cholesterol and MRP1 function (5). Also other ABC transporters have been studied to various extents regarding their dependence on cholesterol, with varying outcomes especially in the case of Pgp (3). Sphingolipids, the other important lipid component of lipid rafts, have not been extensively studied in relation to ABC transporter function. This may be due to the fact that cells have numerous species of sphingolipids, which are not always susceptible to experimental down-regulation. In a previous study we have succeeded in extensive down-

regulation of all sphingolipids in BHK-MRP1 cells, when subjected to a 3 days treatment with myriocin (6). This did not affect MRP1 function as an efflux pump and did not change the ability of MRP1 to associate with lipid rafts. On the contrary, in the present study we show that prolonged myriocin treatment for 7 days did affect MRP1 function, increasing its efflux capacity. This was not related to myriocin-induced reduction of sphingolipid levels, which remained unaltered between 3 and 7 days treatments. Interestingly, various subtle changes in the phospholipid profile were detected in long term myriocin treated cells by mass spectrometry, most importantly increases of several specific lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC) and phosphatidylserine (PS) species. However, rather than promoting MRP1 activity, exogenous addition of these lipids inhibited efflux activity. In fact our data demonstrate that the enhanced activity of MRP1 upon long term treatment with myriocin relates to an enhanced recruitment of MRP1 in lipid raft domains, which promotes its efflux activity.

Material and Methods

Materials

All cell culture plastic was from Costar (Cambridge, MA). Cell culture media, Hank's balanced salt solution, antibiotics, Hepes buffer, L-glutamine and trypsin were from Gibco (Invitrogen, Paisley, UK). Fetal calf serum was from Bodinco (Alkmaar, The Netherlands), [^{3}H]LTC_4 and filter plates were purchased from PerkinElmer (Waltham, MA, USA). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Myriocin was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). The rat monoclonal anti-MRP1 (MRPr1) antibody was obtained from Alexis (Dedham, MD, USA). Prestained protein marker was from New England Biolabs (Ipswich, MA. USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The baby hamster kidney (BHK) cell line stably expressing the human MRP1/ABCC1 gene, named BHK-MRP1, was a gift from Dr. Riordan (Mayo Clinic Arizona, S.C. Johnson

Medical Research Center, Scottsdale, AZ, USA (7). Cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium/NUT mix F-12 (1:1) supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, under standard incubator conditions (humidified atmosphere, 5% CO_2, 37 ^oC). The BHK-MRP1 cells were kept under selective pressure by growing them in the presence of 100 µM methotrexate.

Detection of MRP1-mediated efflux by flow cytometric analysis

BHK-MRP1 cells were plated to confluence in 25 cm^2 flasks one day prior to the experiment. MRP1-mediated efflux of the substrate CFDA was analyzed by flow cytometry as described (2). Alternatively, the efflux of R123 by Pgp was measured using NIH 3T3 MDR G185 cells in a similar fashion.

Isolation of detergent-free lipid raft

Detergent-free lipid rafts were isolated as described (6).

Immunoblot analysis

Protein from equal volumes of the gradient fractions was processed as described (6).

Sphingolipid depletion

In order to deplete the sphingolipid content, cells were grown in the presence of 0.5 µM myriocin for 3 days (short term) or 7 days (long term). In order to be able to isolate membrane vesicles, after 7 days the cell culture was up scaled by growing cells in roller bottles for 3 days (in the presence of myriocin). The sphingolipid content was determined by liquid chromatography mass spectrometry (LC-MS) as described (6). Control cells were incubated and washed similarly as the myriocin treated cells.

Isolation of membrane vesicles from BHK-MRP1 cells

BHK-MRP1 cells were washed with HBSS, trypsinized, harvested and centrifuged (3000 rpm, 3 min), frozen in liquid nitrogen and stored in -80 °C. Pellets of ~ $8x10^8$ frozen cells were used to isolate membrane vesicles as described (5).

Measurement inside-out vesicle ratio

Determination of inside-out vesicle ratio was based on 5`nucleotidase activity and performed as described (5).

Measurement of MRP1-mediated ATPase and transport activities

Vanadate sensitive ATPase activities were measured as described (5). For graphic representation, values were normalized to maximal activity in control conditions (=100%). For transport of LTC_4 into membrane vesicles, vesicles were incubated as described (5). For graphic representation, values were normalized to maximal activity in control conditions (=100%).

Preparation of LPE

1-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine (22:6 LPE) and 1-diarachidonoyl-sn-glycero-3-phosphoethanolamine (20:4 LPE) were generated by treating 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine (22:6 PE) and 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine (20:4 PE), respectively, with phospholipase A2 (8). Six milligrams of 22:6 PE or 20:4 PE was incubated with 0.2 mg phospholipase A2 from Naja mossambica mossambica in the presence of 200 mM Tris, pH 8, 10 mM $CaCl_2$ and 3 mg/ml sodiumdeoxycholate at 37 ^oC for 2 h. The lipid was extracted with chloroform: methanol:acetic acid (50:50:1) and purified by thin-layer chromatography with chloroform: methanol:water (65:25:4) as the mobile phase. Chloroform:methanol (1:1) was used to extract LPE from the silica. On the extracted LPE a phosphate determination was performed to determine the concentration.

Loading membranes with PS, PC or LPE

Cell membranes were loaded with LPE as follows: an appropriate amount of LPE in chloroform was dried (LPE 20:4 and LPE 22:6 species were used), then dissolved in 5 µl ethanol. This LPE solution was added to $1.5x10^6$ cells in 750 µl of HBSS. The final concentration of LPE was 5, 10 or 20 µM, which is equivalent to 12.5x, 25x, or 50x, respectively, of the concentration of endogenous LPE. Cells were incubated for 1 h at 4 °C with LPE. To obtain membrane vesicles with increased PS or PC levels, cells were incubated with methyl-β-cyclodextrin filled with DOPS 18:1 or DOPC 18:1 as described earlier (5) for 30 or 60 min in serum-free medium at 37 °C (10 mM concentration), and subsequently membrane vesicles were prepared as described above.

To load membrane vesicles with LPE, 100 or 200 nM LPE 20:4 or LPE 22:6 (which is equivalent to 40x or 80x, respectively, of the concentration of endogenous LPE) in chloroform/methanol solution was dried, dissolved in 10 µl ethanol and added to 500 µg membrane vesicles in 610 µl (final volume) of isotonic buffer. Membrane vesicles were incubated for 1 h at 37 °C, centrifuged (30000 rpm, 45 min, 4 °C) and subsequently resuspended in isotonic buffer.

Quantification of glycerophospholipids

Quantification of glycerophospholipids was achieved by the use of a LC-MS technique employing synthetic (non-naturally occurring) diacyl and lysophospholipid standards as described (6).

Statistical analysis

Statistical analysis was performed using Student's t-test, with values of $P < 0.05$ indicating statistical significance.

Results

Depletion of sphingolipids with myriocin

In intact BHK-MRP1 cells, both short term (3 days) and long term (7 days) treatments with myriocin yielded the same extent of sphingolipid depletion, i.e. 22.1±1.6 % of control and 23.0±1.9 % of control, respectively (see also ref. 6). In addition, membrane vesicles derived from BHK-MRP1 cells incubated long term with myriocin showed a similar extent of depletion, i.e. 22.7±6.0 % of control.

MRP1 shows increased activity in long term myriocin treated BHK-MRP1 cells

In order to determine whether long term (7 days) myriocin treatment, in contrast to short term treatment (3 days) affected the function of MRP1, we tested MRP1-mediated CFDA efflux activity. BHK-MRP1 cells were loaded with CFDA, and efflux activity was determined on the basis of fluorescence retention in the cells, monitored over a time span of 10 min at 37 °C. Treatment of the CFDA-loaded cells with the MRP1 inhibitor MK571, used as a positive control for inhibition of MRP1-mediated efflux activity, showed a CFDA retention of more than 90 % over a 10 min time interval (Fig. 1A,B). Interestingly, compared to the untreated cells (Fig.1A, continuous line), in long term myriocin treated cells MRP1 appeared substantially more active (Fig. 1A, dotted line). Thus, compared to control cells, after 5 min of efflux time, an approximately 50 % enhanced net efflux of CFDA was seen in the treated cells. Remarkably, short treatment of the cells with myriocin did not affect efflux, its kinetics being identical to that of the control (6 or Fig. 1B). Our data therefore indicate that long term myriocin incubation did enhance the MRP1 activity in contrast to short term treatment, although the sphingolipid content was not further lowered compared to short term myriocin treatment. To investigate whether this increase in MRP1 activity is specific, the same experiment was performed using NIH 3T3 MDR G185 cells, over expressing Pgp. The data clearly demonstrate that contrary to MRP1, P-glycoprotein-mediated efflux activity was not sensitive to myriocin treatment, but was readily inhibited by the Pgp inhibitor CSA, used as a positive control (Fig. 1C).

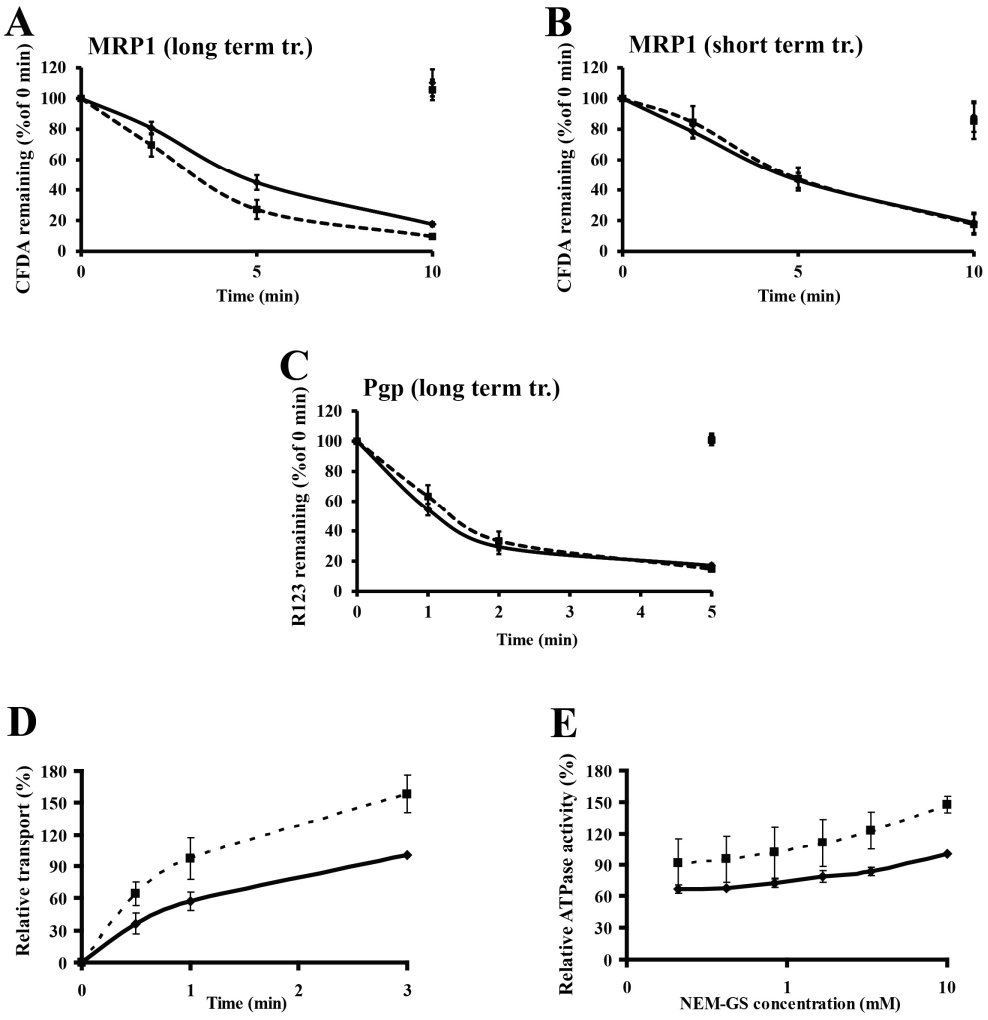

Figure 1. Long term myriocin treatment increases the activity of MRP1
A,B: Efflux assays were performed in long term (A) versus short term (B) myriocin treated and their respective controls using CFDA as a substrate for MRP1 in BHK-MRP1 cells. **C**: Efflux assay in long term myriocin treated and control NIH 3T3 MDR G185 cells using R123 as substrate for Pgp. The Y axis represents the remaining fluorescent substrate relative the starting value at t = 0 min (= 100 %). Values are expressed as the mean ± S.D. (n=3). At 10 minutes also the inhibitor controls are presented, using MK571 for MRP1 and CSA for Pgp. These values range between 85-100 %. **D,E**: vesicular transport of LTC_4 (D) and ATPase activity of MRP1 (E) in isolated membrane vesicles generated from BHK-MRP1 cells. Values are expressed as mean percentage relative to maximal activity of the control ± SD (n=9). In all panels: ♦ solid line: control, untreated; ■ dotted line: long term myriocin treated cells or membrane vesicles derived from these cells.

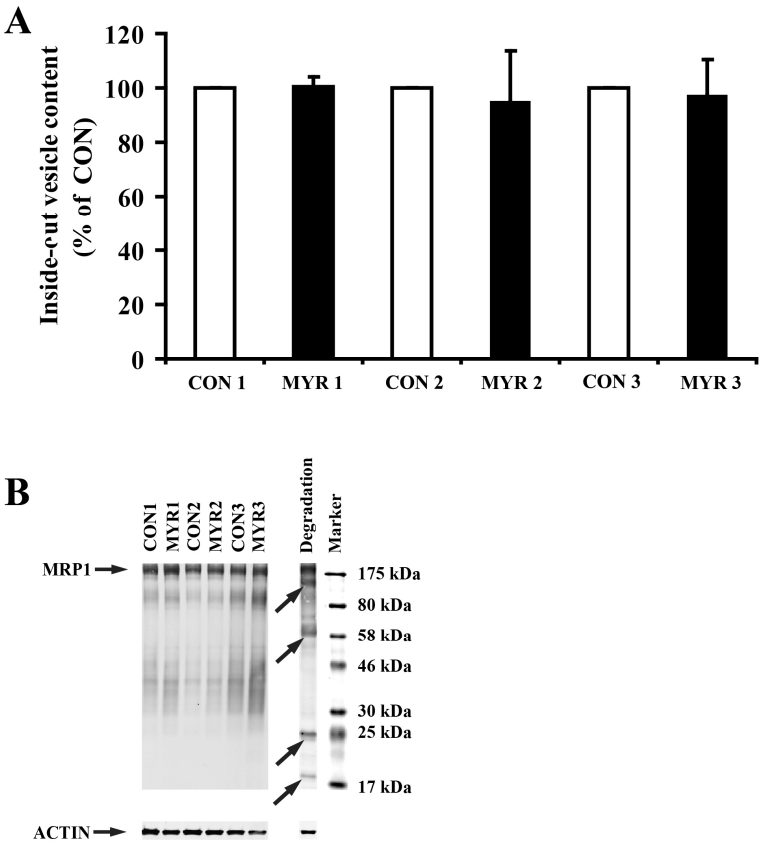

Figure 2. Increased MRP1 activity is not an artifact of variable inside-out vesicle ratio or protein degradation
A: Inside-out vesicle content of control (triplicate white columns) and myriocin treated samples (triplicate black columns). Values are expressed relative to control (=100%) and represent the mean ± SD of three independent measurements.
B: Immunoblot analysis of isolated membrane vesicles shows that MRP1 is intact. Myriocin treatment was performed independently in triplicate and of all samples 10 µg of protein was loaded on the gel. The sample named "Degradation" is a positive control for degradation of MRP1, which was generated by cholesterol-oxidase treatment for 30 min at 37 ºC and shows fragments of MRP1. The marker bands are from top to the bottom 175, 80, 58, 46, 30 25 and 17 kDa.

MRP1 shows increased activity in membrane vesicles isolated from long term myriocin treated cells

The effect of myriocin on MRP1 function could have been due to confounding factors in the complex background of the intact cell.To overcome the complexity of the cell system,

plasma membrane vesicles were isolated from myriocin treated BHK-MRP1 cells. To make sure that myriocin treatment did not affect the inside-out ratio of MRP1 in the isolated membrane vesicles, we measured this ratio. Differences in the inside-out ratio would result in both differences in substrate transport rates across the vesicular membrane and ATPase activity, simply because of differences in MRP1 accessibility in the assay. However, the inside-out vesicle ratios, determined as described in Experimental procedures, were found to be very similar in myriocin and control samples (Fig. 2A). The control inside-out vesicle content was 22.8±3.5 %. Moreover, as verified by immunoblot analysis (Fig. 2B), no significant degradation of MRP1 could be detected, implying that MRP1 integrity was not changed by myriocin treatment.

In this simplified system for MRP1 function analysis, we subsequently measured transport of LTC_4 into the membrane vesicles by MRP1 as well as its ATPase activity (Figs. 1D,E). Both parameters of MRP1 activity showed substantially higher values in membrane vesicles isolated from myriocin treated cells than in such vesicles, derived from control cells (Figs. 1D,E and Table 1). Accordingly, these data are entirely consistent with those presented for the intact cells. Michaelis-Menten kinetic analysis of LTC_4 transport in the vesicular model system revealed that while the K_m values were similar, the V_{max} value of myriocin treated samples was nearly twofold higher than that of the control (Table 1).

	Control	Myriocin treatment	P value
ATPase activity (nmol Pi/mg/min)	27.4 ± 3.8	40.2 ± 7.8	**0.004**
Vesicular transport (pmol/mg/min)	25.9 ± 6.3	44.8 ± 11.0	**0.002**
K_m (nM)	628.6 ± 236.7	706.1 ± 214.7	0.602
V_{max} (pmol/mg/min)	225.4 ± 43.2	457.0 ± 101.8	**0.001**

Table 1. MRP1 activity in isolated membrane vesicles
BHK-MRP1 cells were long term myriocin treated, and subjected to the membrane vesicle isolation procedure. Three independent membrane preparations were generated. For each membrane preparation, the measurements were performed in triplicate. The table is a summary of the results of the ATPase activities, the vesicular transport assays and the Michaelis-Menten kinetic analysis performed on the vesicular transport data. Data indicate the mean ± SD of 9 independent measurements.

The enhanced activity of MRP1 after long term myriocin treatment could be due to upregulation of MRP1 levels during this treatment. However, the levels of MRP1 in treated versus control intact cells as well as in the cell-derived membrane vesicles were found to be similar. Thus the ratio of the level of MRP1 normalized to actin in treated versus control intact cells was 1.20±0.38 (n=22; P=0.64). For membrane vesicles this ratio was 1.03±0.11 (n=18; P=0.65).

MRP1 shows increased association with lipid rafts in long term myriocin treated cells

We have previously shown that MRP1 is enriched in lipid rafts (2,6). Here we tested the effect of long term myriocin treatment on the association of MRP1 with these domains, using the detergent-free method for isolation of lipid rafts. Gradient fractions 1-2 as well as fractions 3-4 obtained with this method contain floating membranes, which are highly enriched in cholesterol and sphingolipids and have very low protein content, thus fitting the criteria for lipid rafts (6). Intriguingly, following a long term treatment of the cells with myriocin, MRP1 showed a significantly higher extent of association with the lipid raft fractions 3-4 (Fig. 3A, black bars) than in control cells (white bars). This increase represents a shift from the non-raft fractions 7-9, indicating recruitment of MRP1 from non-raft membrane to lipid raft domains. A very similar pattern was obtained for the lipid raft marker caveolin (Fig. 3B). However, the increase of another lipid raft marker Src in fractions 3-4 was not significant (Fig. 3C).

Myriocin treated cells show slight differences in glycerophospholipid profile

It is conceivable that in response to the loss of sphingolipids after long term myriocin treatment compensatory mechanisms might be triggered as a consequence of which the glycerophospholipid profile may change. Therefore LC-MS was used to analyze the glycerophospholipids in terms of headgroup and fatty acid composition as well as the total amount of this lipid pool. However, we found no significant changes in the total glycerophospholipid content or in the content of phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylinositol (PI) after long term myriocin treatment.

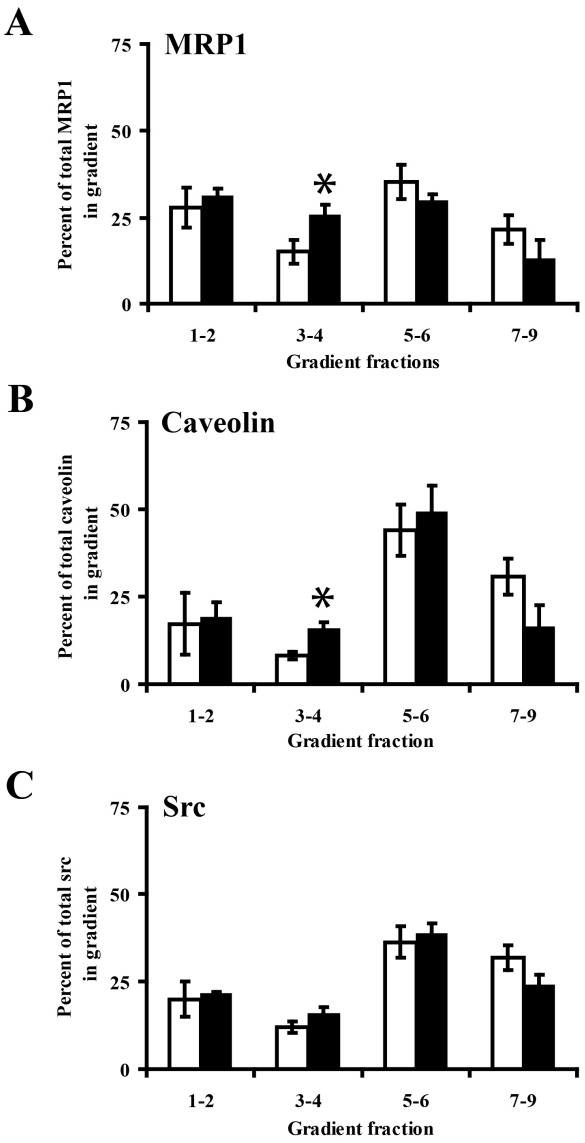

Figure 3. Effects of myriocin treatment on detergent-free lipid raft localization of MRP1, caveolin and Src in BHK-MRP1 cells

A-C: BHK-MRP1 cells were long term treated with myriocin and lipid raft association of MRP1 was determined under these conditions. The values indicate the percentage of MRP1 (A), caveolin (B) and Src (C) found in the gradient fractions, relative to total amount in the entire gradient (9 fractions). White bars, control cells; black bars, myriocin-treated cells. Data represent the mean ± SD (n=3). Asterisks indicate values that are significantly different from control ($P < 0.05$, as determined by Student's t-test).

Neither were changes observed in the content of the lyso-forms of PA, PG and PI (data not shown). However, we did observe changes in the total content of distinct LPE, PS and PC species, which were summarized in Table 2.

Lipid	Fold	P value
LPE 18:1	1.92	0.320
LPE 20:4	4.92	0.126
LPE 20:1	2.26	0.241
LPE 22:5	4.12	0.144
LPE sum	2.26	0.320
PS 40:2	1.25	**0.001**
PS 40:4	1.63	**0.001**
PS 40:5	1.43	**0.000**
PS sum	1.03	0.586
PC 32:0	1.34	**0.000**
PC 34:0	1.11	**0.027**
PC 36:1	1.09	**0.020**
PC sum	1.02	0.447

Table 2. Major changes in lipid species after long term myriocin treatment
LC-MS was used to analyze the glycerophospholipid composition of the cells in terms of headgroup and fatty acid composition. Glycerophospholipids are indicated with the sum of C-atoms of the two fatty acids and the total number of double bonds. Fold indicates the ratio of the amount of a given glycerophospholipid in myriocin treated samples divided by the amount in control samples. The table gives an overview of those lipid species which were different from control after long term myriocin treatment. The sum of all lipid species in a certain lipid class is also indicated. Data represent the mean of 3 independent experiments.

Several relatively long-chain LPE species appeared to be more abundant in long term myriocin treated cells, although the high fold values did not reach statistical significance. In this context it should be mentioned that the LPE contributes only 2-5% of the total lipid mass (data not shown). PS and PC together contribute about 60% of the total lipid mass. Although the total content of PS or PC species did not differ from control (Table 2), in both these lipid classes several species were more abundant in long term myriocin treated cells. However, both the absolute and relative overall lipid composition of raft fractions isolated from long term myriocin treated cells did not show any statistical differences compared to control (data not shown).

Loading cell membranes with LPE or loading membrane vesicles with LPE, PS, or PC does not increase MRP1 activity

We reasoned that if the enhanced activity of MRP1 after long term myriocin treatment is a direct effect of increases in one or more of the lipids as reported in the previous paragraph, a similar effect might be achieved by their exogenous addition, Accordingly, we examined the effects of exogenous addition of LPE, PS and PC on MRP1 activity in control and myriocin treated cells and membrane vesicles. First, we loaded control cells and isolated membrane vesicles derived from control cells with LPE. However, the efflux activity of MRP1 measured in intact cells actually decreased with stepwise increases of the LPE concentration used for loading of the cells (Fig. 4, white bars). Membrane vesicles derived from control BHK-MRP1 cells loaded with LPE showed a similar effect; both vesicular transport and vanadate sensitive ATPase activity were stepwise reduced with increasing concentrations of LPE (Fig. 5A,B).

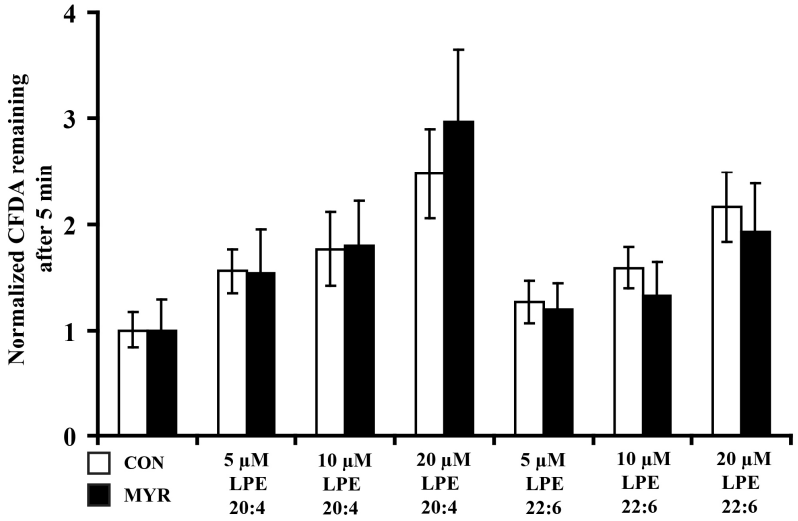

Figure 4. LPE loading of BHK-MRP1 cells decreases the efflux activity of MRP1
Control cells (white bars) and short term myriocin treated cells (black bars) were loaded with LPE 20:4 or LPE 22:6 at 5, 10 or 20 µM concentrations for 1 h at 4 ºC. The same experiment was done at 37 ºC and gave similar result (data not shown). The cells were allowed to efflux for 5 min. Data represent the remaining fluorescence of CFDA relative to the 0 min value and normalized to control (=1) or myriocin treated cells (=1), respectively. Data are the means of three independent experiments ± SD.

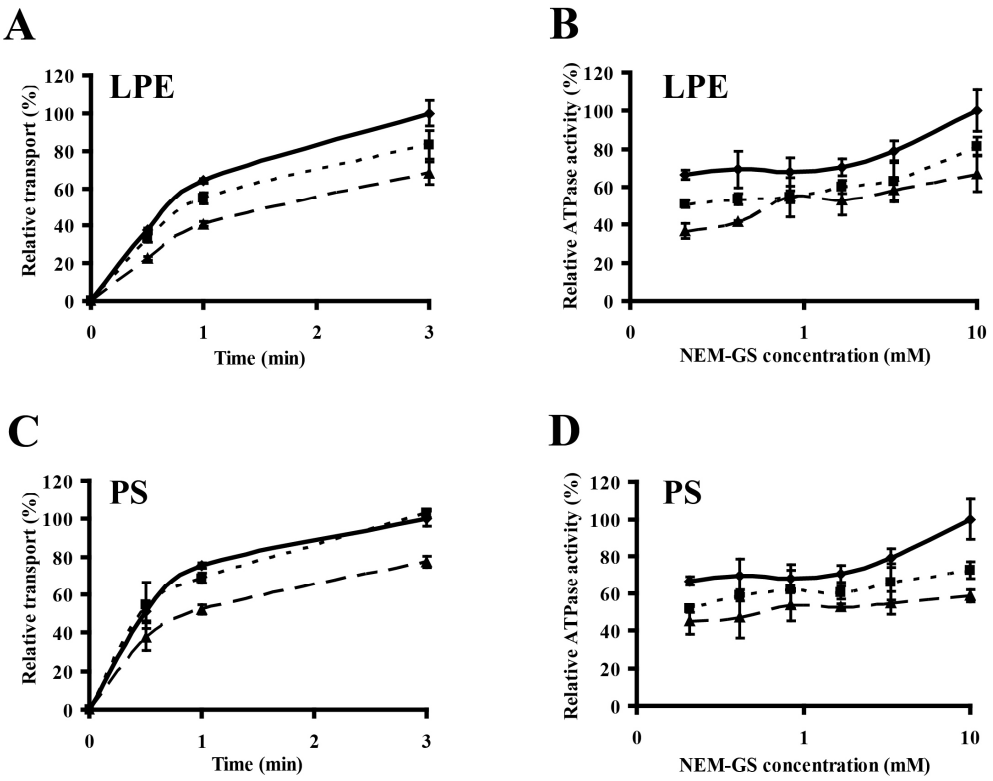

Figure 5. Loading the membrane with LPE or PS does not increase the activity of MRP1 in membrane vesicles
A,B: Membrane vesicles derived from control BHK-MRP1 cells were loaded with LPE 20:4 or LPE 22:6 (the latter data not shown because of similarity). **C,D**: Intact control BHK-MRP1 cells were loaded with DOPS, followed by membrane vesicle isolation. Vesicular transport (A,C) or vanadate sensitive ATPase activity (B,D) of MRP1 in these vesicles were measured. In all panels: ♦ solid line: control, ■ dotted line and ▲ dashed line: stepwise increased loading of lipids. Values are expressed as mean percentage relative to maximal activity of the control ± SD (n=3).

To properly evaluate the potential effect of the phospholipids following myriocin treatment, short term treated cells (3 days) were used as well, because they have very similar sphingolipid levels as 7 days treated cells. In these short term treated cells, potential phospholipid-induced changes in MRP1-mediated efflux upon their exogenous addition, might best simulate those triggered inherently after the long term treatment. Similarly as in control cells we observed an inhibition of MRP1 efflux activity (Fig. 4, black bars).

Next we focused on a potential effect of PS. We therefore prepared PS-enriched membrane vesicles from control cells that had been treated with DOPS filled methyl-β-cyclodextrin. Table 3 shows the efficiency of this procedure. The lipid loaded vesicles were indeed enriched in the specific lipid, although the results suggest that during the procedure the double bond in the acyl chain is susceptible for oxidation. However, these PS enriched membrane vesicles did not show enhanced MRP1 transport or vanadate sensitive ATPase activities (Fig. 5C,D). Rather, a decreased activity is measured as was the case when LPE was added. Similar results, i.e. a decreased activity of MRP1, were obtained when membrane vesicles were loaded with DOPC (data not shown).

	PS 30 min		PS 60 min	
	Fold	P value	Fold	P value
PS 36:2	1.85	**0.000**	1.38	**0.000**
PS 36:1	1.44	**0.002**	1.32	**0.003**
PS 36:0	2.87	**0.009**	2.33	**0.034**

Table 3. PS content of DOPS loaded membrane vesicles
BHK-MRP1 cells were loaded with DOPS by incubating them with lipid loaded methyl-β-cyclodextrin for 30 or 60 min at 37 °C. Subsequently membrane vesicles were prepared and analyzed for glycerophospholipids by LC-MS. Glycerophospholipids are indicated with the sum of C-atoms of the two fatty acids and the total number of double bonds. Only the relevant ones are shown. Fold indicates the ratio of the amount of a given glycerophospholipid in myriocin treated samples divided by the amount in control samples. Data represent the mean of 3 independent measurements.

Discussion

In this study we show that MRP1 activity as an efflux pump is increased after long term (7 days) myriocin treatment. This was demonstrated in intact BHK-MRP1 cells with a CFDA efflux assay, and corroborated in membrane vesicles isolated from long term myriocin treated cells. The latter results are important, as they show that the myriocin effect is not a result of confounding factors in the complex system of the intact cell. Rather, an increase of MRP1 activity is also apparent in relatively simple membranes containing the transporter. We have previously shown that short term (3 days) myriocin treatment did not affect MRP1 function (6), in spite of the fact that the residual cellular levels of sphingolipids after either 3 or 7 days treatment are very similar with regard to both content and composition. The

long term myriocin effect can therefore not be attributed to a further loss of sphingolipids or an alteration in sphingolipid composition.

We therefore further investigated potential mechanisms underlying the long term myriocin effect. An increased expression (upregulation) of MRP1, differences in the protein's stability (occurrence of degradation) or a different inside-out ratio of the transporter in the membrane vesicle system would be obvious reasons for differences in MRP1 activity in treated versus non-treated cells. However, these options were all experimentally excluded. We have previously observed that the extent of lipid raft association of MRP1 is an important factor for its activity (2), reflecting the relevance of a particular environment for optimal expression of the transporter's activity. Therefore we studied the lipid raft association of MRP1 at conditions following long term myriocin treatment. Interestingly, our data revealed a significant increase in the association of MRP1 to the lipid raft fractions 3-4 (Fig. 3). More specifically, the results indicate a shift from fractions 7-9 to fractions 3-4 under these conditions. These observations thus suggest that the total amount of MRP1 in a raft environment is increased and this may (partially) explain the higher efflux activity measured. The basis for increased activity of MRP1 in lipid rafts is not understood. However, it does not appear to be related to the lipid composition of the rafts. We speculate that MRP1 needs a certain physical environment, i.e. a more tightly packed lipid surrounding, for its optimal conformation.

A particular advantage of the membrane vesicle system that we exploited in this study is that it allows us to perform Michaelis-Menten kinetic analysis on the LTC_4 transport data. This showed that the K_m value in the case of long term myriocin treatment was similar to control, which indicates that the intrinsic transport activity of single MRP1 molecules was not changed. In contrast, the V_{max} value nearly doubled and this indicates that there is a larger fraction of active transporter molecules under conditions of long term myriocin treatment. This finding and interpretation is entirely consistent with the observation that there is a higher extent of lipid raft association of MRP1, a condition that promotes MRP1 activity.

In this context, having excluded in previous work a particular role of sphingolipids (6) and cholesterol (5) on MRP1 activity, we therefore focused on a potential effect of

phospholipids, taking also into account that specific phospholipids could act as allosteric regulators of MRP1-function (9). Indeed, using LC-MS we established that several changes in certain species of glycerophospholipids had occurred, involving the lipid classes LPE, PS and PC. If these lipids would affect MRP1 function, it seems reasonable to assume that their exogenous addition to control cells might mimic their potential effect on MRP1. However, quite unexpectedly, instead of an enhancement of MRP1 activity, a substantial decrease in its activity was observed upon exogenous addition of LPE, PS or PC to both myriocin-treated and untreated cells. This effect could not have resulted from a complex interplay between exogenously added glycerophospholipids and endogenous sphingolipids, because a similar decrease of MRP1 function was observed when glycerophospholipids were added to myriocin treated, and thus sphingolipid depleted, cells. Rather than being due to allosteric effects of phospholipids on MRP1 structure, we interpret the negative effect of the exogenously added phospholipids on MRP1 function to result from a disturbance of the integrity of the MRP1-localizing membrane domains, including rafts, since ABC transporters are known to be affected in their function by lateral membrane perturbations (10). Indeed, it has been well-established that exogenous inclusion of lysolipids and or (saturated) phospholipids in the lateral plane of a bilayer may trigger local curvature changes, alterations in membrane hydration or modulate catalytically important motions of proteins, thereby negatively affecting protein function (11,12). Moreover, our observations in the membrane vesicle model system, showing that the enhanced activity of MRP1 in long term treated cells relates to a change of Vmax rather than Km, further excludes the possibility of the involvement of allosteric effects of specific glycerophospholipids on MRP1 function.

In conclusion, we have shown that long term myriocin treatment increases MRP1 activity as an efflux pump. This effect is not related to depletion of sphingolipids, but rather to an increased association of MRP1 with lipid rafts, while a significant contribution of allosteric interactions with specific glycerophospholipid species, potentially promoting MRP1 activity, could be excluded. The use of the membrane vesicle system to study MRP1 function serves two purposes: 1. The data confirm the conclusions drawn from experiments with intact cells, and exclude confounding factors in the complex situation of the intact cell.

2. We provide an opportunity to experimentally enhance the activity of MRP1 in a model system that is widely used for ABC transporter studies and this may be useful to others working with these systems.

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Chapter 5

Actin dependence of the closely related MRP1, MRP2, MRP3 and MRP5 differs in MDCKII cells

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Abstract

Multidrug resistance of cancer cells is a serious obstacle towards successful chemotherapy. The overexpression of ABC transporters appears to be the main mechanism of MDR and MRP1 is one of the three most important proteins in this context next to Pgp and BCRP. Previously our group has shown that MRP1 localization in lipid rafts as well as its efflux activity strongly depends on cortical actin. In the present paper we compare the actin dependence of MRP2, MRP3 and MRP5 to MRP1, separately expressed in MDCKII cells as a model system. The closely related MRPs were found to respond differently upon disruption of the actin cytoskeleton. The efflux activity of MRP2 was decreased, but MRP1-mediated efflux was unaffected, as was the case for MRP3 and MRP5. The presence or absence of an effect on MRP-mediated efflux activity fully correlated with the presence or absence, respectively, of a shift of the respective MRP out of lipid rafts, which occurred only for MRP2. In conclusion, in MDCKII cells actin disruption reduces both efflux activity and lipid raft association of MRP2, but not MRP1, MRP3 and MRP5.

Introduction

Chemotherapy is still the only treatment possible for cancer patients with disseminated disease. Unfortunately, during the course of treatment, cancer cells often develop resistance against anticancer drugs, or even multidrug resistance against a large array of drugs. One of the mechanisms causing MDR is the overexpression of ABC transporters, which protect the tumor cell by pumping out a wide range of structurally different drugs.

The ABC transporter family is largest one among the transmembrane protein families. 49 ABC coding genes have been identified in the human genome, but they are present in all known organisms from bacteria to humans and are divided into 7 subfamilies (A-F) based on amino acid sequence similarities.

The first member of the C family, MRP1, is one of the three most important transporters playing role in MDR next to Pgp and BCRP (1). Previously MRP1 has been shown to be localized in lipid rafts. Localization of MRP1 in lipid rafts on the cell surface as well as its function as an efflux pump were found to depend on actin in Neuro-2a and BHK-MRP1 cells (2). We next wondered whether this behavior is specific for MRP1 or occurs cell type specific and whether this behavior extends to other MRP family members or not. In this study we therefore systematically investigated MRP1, MRP2, MRP3 and MRP5 in one and the same cell background, i.e. MDCKII cells in which these transporters had been separately transduced. We show that in contrast to Neuro-2a and BHK-MRP1 cells, MRP1 is insensitive to actin disruption in MDCKII cells. However, MRP2 efflux activity as well as its localization in lipid rafts was both reduced by actin disruption. Among the MRPs tested, only MRP2 showed sensitivity towards actin disruption, while MRP3 and MRP5 were unaffected like MRP1.

Materials and methods

Materials

All cell culture plastic was from Costar (Cambridge, MA, USA) and liquids from Gibco (Invitrogen, Paisley, UK). Fetal calf serum was from Bodinco (Alkmaar, The Netherlands).

Latrunculin B, cytochalasin D, nocodazole, the monoclonal anti-β-actin antibody, probenecid and sulfinpyrazone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 488-conjugated phalloidin and Alexa Fluor-conjugated secondary antibodies were obtained from Molecular probes (Eugene, OR, USA). OptiPrep was from Axis-Shield PoC AS (Dundee, Scotland). The polyclonal anti-Cav-1 antibody and the monoclonal anti-Rho-GDI antibody were from Transduction Laboratories (Lexington, KY, USA). The polyclonal anti-c-Src antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The monoclonal anti-MRP1 antibody was from Alexis Biochemicals (Lausen, Switzerland). The monoclonal anti-MRP2 (M3III-6) antibody was from Enzo Life Sciences (Zandhoven, Belgium). The monoclonal anti-MRP3 antibody was from Chemicon International Inc. (Millipore) (Billerica, MA, USA). The monoclonal anti-MRP5 (M5I-1) antibody was from AbCam (Cambridge, UK).

Cell culture and incubation conditions

Parental MDCKII cells (PAR) and those retrovirally transduced with MRPs (MDCKII wild-type, 3), MDCKII-MRP1 (5,6,7,27), MDCKII-MRP2 (4,6,8), MDCKII-MRP3 (7), and MDCKII-MRP5 (9) were kindly provided by Alfred H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). These cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, under standard incubator conditions (humidified atmosphere, 5% CO_2, 37 °C). The cells were never grown to confluence or in the presence of any drugs. Moreover, the cell cultures were never maintained beyond 2 months of culture and were always trypsinized 2 days prior to experiments.

In order to disrupt the actin cytoskeleton, cells were treated with cytochalasin D (10 µg/ml) or latrunculin B (10 µM) for 45 minutes. In some cases, cells were preincubated with nocodazole (10 µM) for 45 minutes to disrupt microtubules, followed by treatment with both nocodazole and latrunculin B for 45 minutes. Drugs were also included during the trypsinization of cells prior to experiments. All incubations with cytoskeleton modulators and all control experiments without these modulators were performed in medium with serum.

Isolation of detergent-free lipid rafts

MDCKII-MRPs cells were cultured to 50-70 % confluence in 162 cm^2 flasks and treated with cytoskeleton modulators as mentioned above. Detergent-free lipid rafts were isolated as described (10,11).

Immunoblot analysis

Protein from equal volumes of the gradient fractions was TCA-precipitated and resuspended in sample buffer (5% SDS, 5% β-mercaptoethanol, 0.125M Tris-HCl pH 6.8, 40% glycerol). The samples were processed for Western blot as described (11), using primary antibody against MRP1, MRP2, MRP3, MRP5, radixin (all 1:2000).

Detection of MRP-mediated CFDA efflux

MDCKII cells transduced with MRPs were cultured to ~50-70 % confluence in 25 cm^2 flasks and treated with cytoskeleton modulators as mentioned above. Cells were harvested by trypsinization, washed with HBSS and incubated with the substrate CFDA (0.5 µM in HBSS) at 10 °C for 60 minutes. Cells were washed with ice-cold HBSS. Subsequently, the cells were incubated at 37 °C in the presence or absence of the inhibitor MK571 (25 µM in case of MDCKII-MRP1 cells), probenecid (1.5 mM in case of MDCKII-MRP2 and MDCKII-MRP3 cells) or sulfinpyrazone (1.5 mM in case of MDCKII-MRP5 cells) during various time intervals. The efflux of the fluorescent substrate was stopped by mixing the cells with ice-cold HBSS containing the appropriate inhibitors (in the above mentioned final concentrations) and keeping them on ice. The remaining cell-associated fluorescence was determined by flow cytometric analysis using an EliteTM flow cytometer (Beckman Coulter, Miami, FL).

Confocal laser scanning fluorescence microscopy

MDCKII cells transduced with MRPs were grown to 50-70 % confluence on glass cover slips in 12 well plates and treated with cytoskeleton modulators as mentioned above. Cells were processed for confocal microscopy as described (2). Cells were stained with antibodies against MRPs (1:150), Cav-1 (1:150), and appropriate Alexa Fluor-conjugated

secondary antibodies. Alternatively, F-actin was stained with Alexa Fluor 488-conjugated phalloidin (1:150). Analysis of the samples was performed using a TCS Leica SP2 AOBS Confocal Laser Scanner Microscope (Leica, Heidelberg, Germany), equipped with a HCX PL APO 63x 1.4 oil CS objective in combination with Leica Confocal Software. The system was operated in the sequential mode in case of double labelled samples. Images were processed using Adobe Photoshop CS3.

Results

Validation of the model cell lines; expression of MRPs and MRP-mediated efflux

The first objective was to confirm that the MDCKII cell lines express the correct MRPs. Western blotting confirmed the presence of MRP1 in MDCKII-MRP1 cells, MRP2 in MDCKII-MRP2 cells, MRP3 in MDCKII-MRP3 cells and MRP5 in the MDCKII-MRP5 cell line. Furthermore, representative actin bands are shown (Fig. 1A).

Secondly the efflux activities of MRPs in the different cell lines were measured. All four MRPs can transport CFDA (Fig. 1B) and this activity is inhibited by 25 µM MK571 (MRP1, white columns), 1,5 mM probenecid (MRP2, light gray columns and MRP3, gray columns) or 1,5 mM sulfinpyrazone (MRP5, dark gray columns) to roughly 80-95 % of remaining CFDA. We also observed some efflux activity in the parental MDCKII cells. This could be due to endogenous dog Mrp1, since it was inhibited by MK571. However, we could not detect MRP1 in parental cells using Western Blot. Nevertheless, in the MRP2-, MRP3-, and MRP5-containing cell lines a contribution of this parental efflux system should be excluded. First, 48 % of remaining CFDA in parental cells represents a considerably smaller efflux activity than that observed in the transduced (MRP) cell lines (Fig. 1B, black columns). More importantly, the parental activity cannot be inhibited with probenecid or sulfinpyrazone in the same concentrations used before, while the efflux activity in MRP2-, MRP3-, and MRP5-containing cell lines is virtually completely inhibited. If there would be a considerable contribution by the parental efflux system, inhibition by probenecid or sulfinpyrazone would be incomplete.

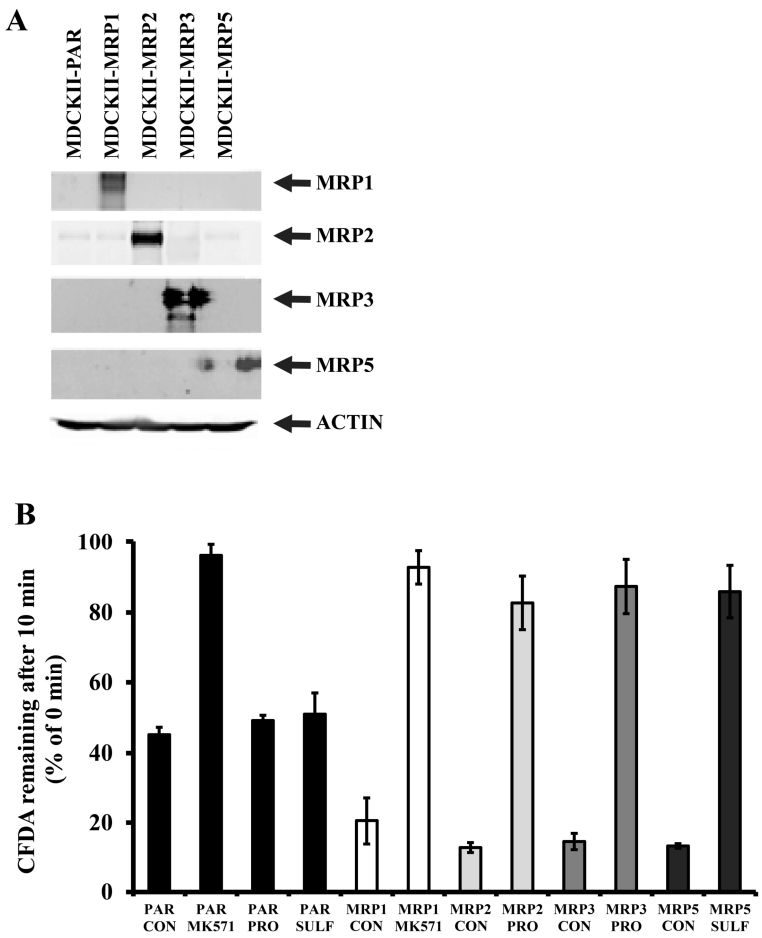

Figure 1. Validation of the model cell line MDCKII
A) Western blotting **B**) Cells were loaded with CFDA and efflux was allowed to proceed for 10 minutes in the presence or absence of inhibitors. MDCKII parental cells (PAR, black columns) show limited efflux of CFDA, which is inhibited with 25 µM MK571, but not with 1.5 mM probenecid (PRO) or 1.5 mM sulfinpyrazone (SULF). The efficient efflux in MDCKII cells transduced with MRP1 (white columns), MRP2 (grey columns), MRP3 (vertically striped columns) and MRP5 (horizontally striped columns) can be strongly inhibited. Data represent the mean +/- SD of 3 independent experiments.

Actin is disrupted by cytochalasin D and latrunculin B

The efficacy of actin cytoskeleton modulators was analyzed by confocal microscopy (Fig. 2). In control cells (Fig. 2A), both stress fibers (arrow) and cortical actin (arrowhead) were observed. Cortical actin stained strongly at the edges of groups of cells.

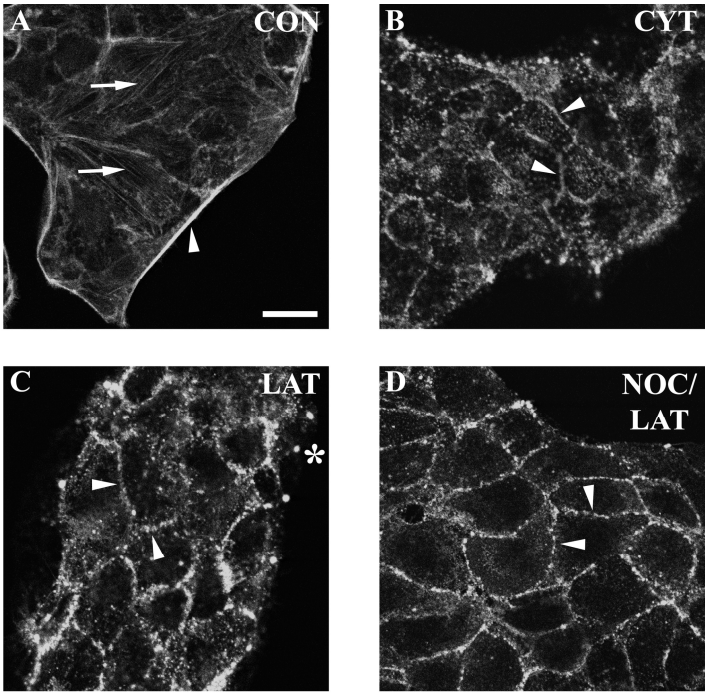

Figure 2. Visualization of actin cytoskeleton in parental MDCKII cells
Cells were untreated (A; CON) or treated with 10 µg/ml cytochalasin D (B; CYT), 10 µM latrunculin B (C; LAT) or 10 µM nocodazole followed by 10 µM nocodazole + 10 µM latrunculin B (D; NOC/LAT). Arrows indicate actin stress fibers, arrowheads cortical actin, asterisk indicates patches of actin. Bar 20 µm.

After cytochalasin D treatment (Fig. 2B), stress fibers were disrupted, while cortical actin was still present (arrowheads), although slightly dotted. Latrunculin B treatment disrupted cortical actin to a larger extent and stress fibers completely (Fig. 2C). At the edges of groups of cells a strong punctate pattern (asterisk) was observed which indicates accumulation of disrupted cortical actin, while cortical actin in cells inside the groups presented as a dotted pattern (Fig. 2C). After both cytochalasin D and latrunculin B treatments, actin also accumulated as patches in the cytoplasm (Fig. 2B,C). When latrunculin B treatment was preceded by nocodazole treatment (NOC/LAT), the actin staining pattern was similar to that in latrunculin B treated cells, but without actin accumulation in the cytoplasm (Fig. 2D).

Actin disruption results in changes in MRP2 localization

Next we analyzed the localizations of MRPs in the transduced MDCKII cell lines (Fig. 3).

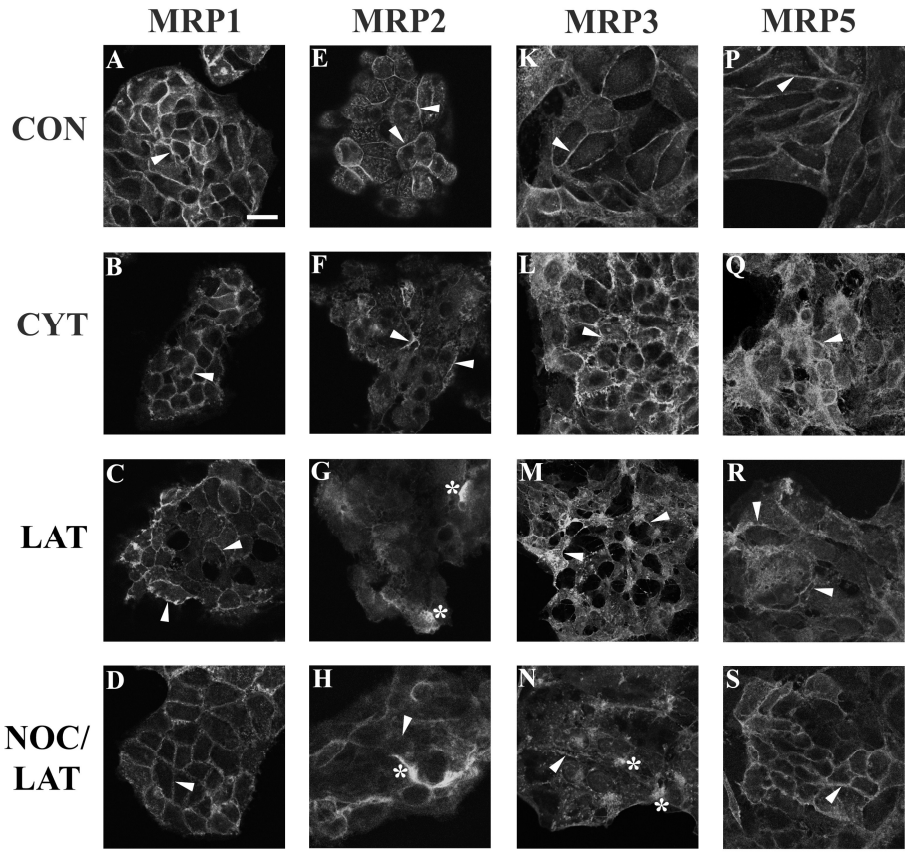

Figure 3. Visualization of MRPs in MDCKII-MRP1, MDCKII-MRP2, MDCKII-MRP3 and MDCKII-MRP5 cells
Cells were untreated (A,E,K,P; CTRL) or treated with cytochalasin D (B,F,L,Q; CYT), latrunculin B (C,G,M,R; LAT) or nocodazole followed by nocodazole + latrunculin B (D,H,N,S; NOC/LAT). Arrowheads indicate the plasma membrane of cells, while the asterisks indicate patches of MRP2 (G,H) or partial internalization of MRP3 (N). Bar 20 µm.

Although the cytochalasin D, latrunculin B and nocodazole treatments had profound effects on cell and membrane morphology, the staining patterns of MRP1, MRP3 and MRP5 remained fairly similar to control conditions. These transporters were found in the plasma membrane (Fig. 3, arrowheads) in all conditions, most clearly in the case of MRP1 (Fig. 3A-D). MRP3 showed some cytoplasmic staining in latrunculin B treated cells pre-treated

with nocodazole (Fig. 3N). The most dramatic changes, however, were observed with MRP2 (Fig. 3E-H). While control cells showed a clear plasma membrane staining pattern (Fig. 3E), this was strongly reduced in cytochalasin D treated cells (Fig. 3F) and virtually absent in latrunculin B treated cells, either with or without nocodazole pre-treatment (Fig. 3G,H). Unlike other MRPs, MRP2 was present in local strongly staining patches with a thickness that does not correspond to the plasma membrane (Fig. 3G,H, asterisks). These patches may represent MRP2 accumulations near the plasma membranes of cells, either on the inside or outside of cells.

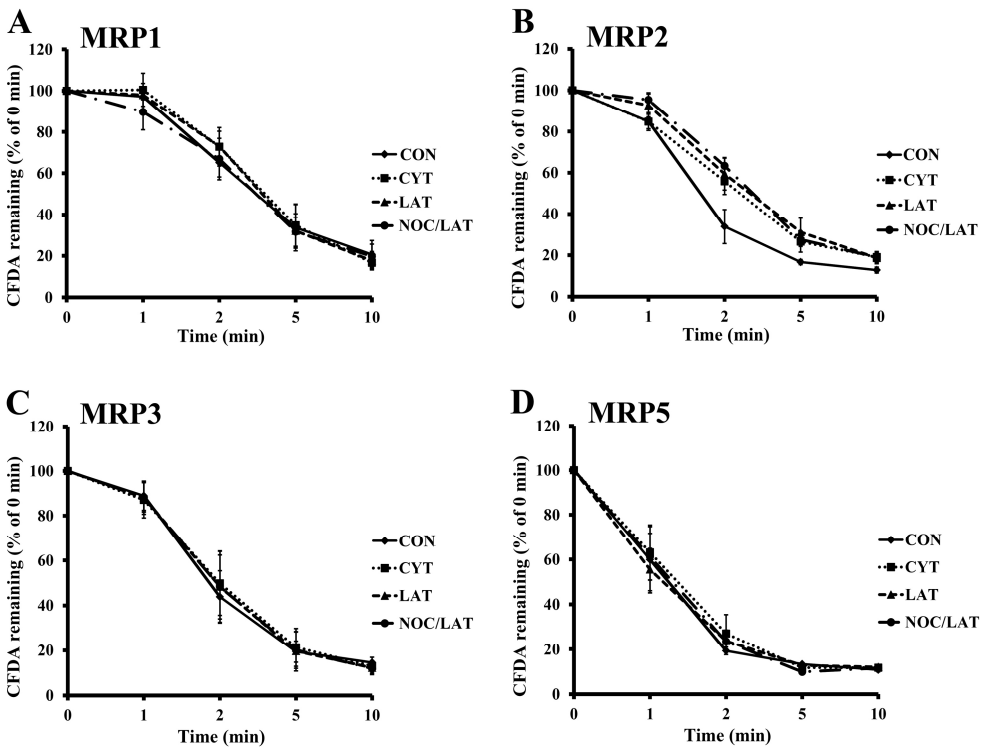

Figure 4. Efflux activities of MRP1, MRP2, MRP3 and MRP5 after actin disruption
Efflux was measured in control cells and cells treated with cytochalasin D (CYT), latrunculin B (LAT) or nocodazole followed by nocodazole + latrunculin B (NOC/LAT). Activity decrease compared to the control can be observed in case of MRP2 in all three actin cytoskeleton modulated conditions (B). For MRP1, MRP3 and MRP5 there are no differences between actin modulation and control. Values are presented as the mean % of 0 minutes +/- SD (n=3).

Actin disruption decreased MRP2-mediated efflux

The changes in MRP2 localization upon treatment with actin modulators as described above were paralleled by changes in MRP2-mediated efflux activity, as measured using CFDA as substrate (Fig. 4B). The effect is most pronounced after 2 minutes of efflux, when the remaining CFDA in treated cells is 60% compared to 34% in control cells. In contrast, the efflux activities of MRP1 (Fig. 4A), MRP3 (Fig 4C) and MRP5 (Fig. 4D) were unaffected by actin disruption.

Actin disruption decreased MRP2 in lipid raft fractions

We investigated the lipid raft association of MRPs using detergent free lipid raft isolation technology. Latrunculin B treatment was selected for this analysis, because this treatment showed the strongest effects on all parameters tested thus far, i.e. actin disruption (Fig. 2), MRP2 localization (Fig. 3) and MRP2-mediated efflux (Fig. 4). Interestingly, MRP2 showed the highest extent of lipid raft association among the tested MRPs under control conditions (Fig. 5B), followed by MRP1 (Fig. 5A), MRP3 (Fig. 5C) and MRP5 (Fig. 5D). Latrunculin B strongly reduced lipid raft association of MRP2 from 47% to 21% (Fig. 5B), but not of MRP1 and MRP5. The effect on MRP3 lipid raft association was highly variable and not significant ($P > 0.05$). We also analyzed lipid raft association of radixin and this was significantly reduced after latrunculin B treatment (Fig. 5E).

Discussion

In this paper we show that in MDCKII cells MRP2 is selectively sensitive to actin disruption among 4 tested MRPs. Both cytochalasin D and latrunculin B treatment cause MRP2 to partially shift out of lipid rafts and lose efflux activity. This shows that actin stabilizes MRP2 in lipid rafts as well as its efflux function. Results suggest that actin stress fibers are responsible for this, since cortical actin remains intact after cytochalasin D treatment, but efflux function of MRP2 decrease. Cytochalasin D and latrunculin B have in common that they fully disrupt actin stress fibers. Unfortunately we cannot induce a condition, where actin stress fibers are intact, while cortical actin is disrupted. This could

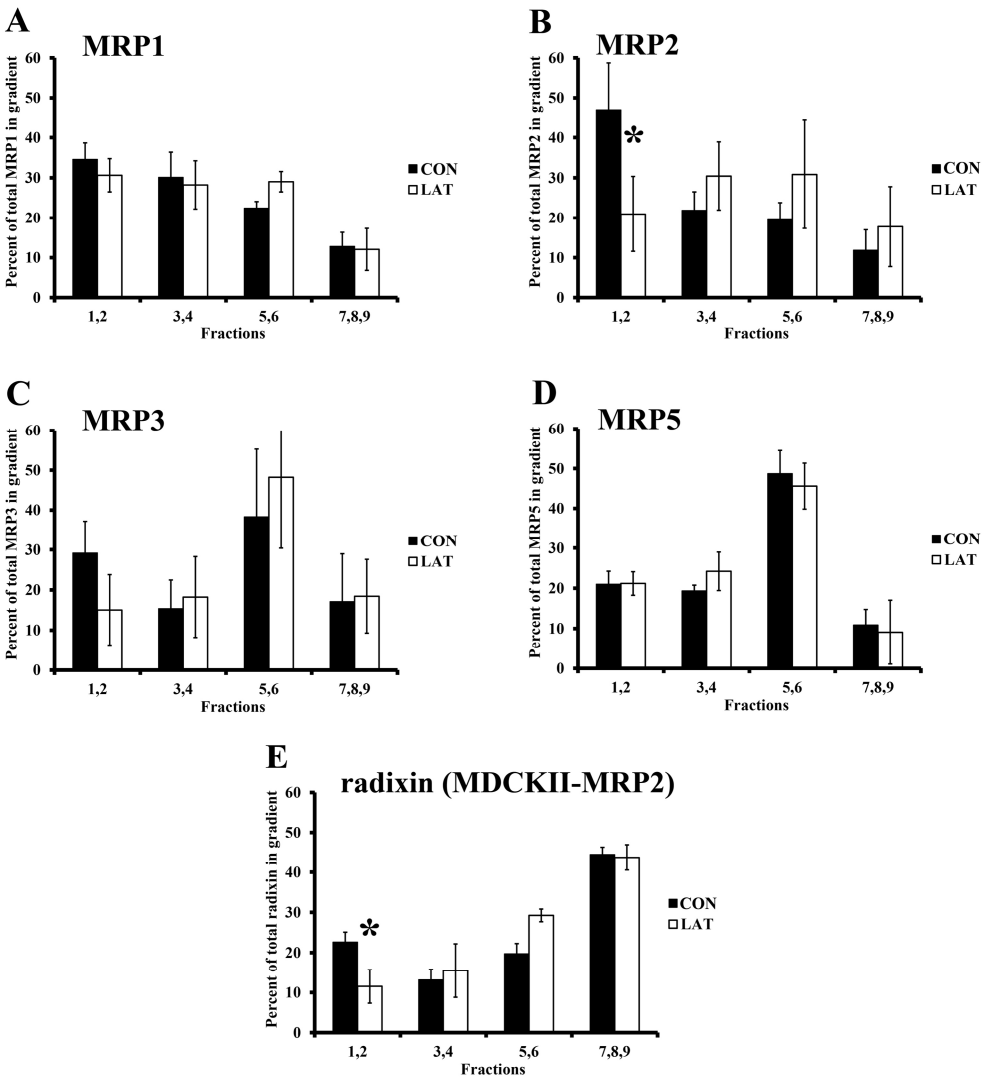

Figure 5. Lipid raft association of MRP1, MRP2, MRP3 and MRP5 after actin disruption
MRP2 (B) and radixin (E) shifted out of the lipid raft fractions 1,2 after latrunculin B treatment (*Values of fractions 1,2 are significantly ($P < 0.05$) different between LAT and CON as determined by Student's t-test). There were no significant differences between LAT and CON conditions for MRP1 (A), MRP3 (C) and MRP5 (D). The numbers indicate the percentage of a specific protein found in pooled gradient fractions, relative to the total of that protein in the entire gradient. Data represent the mean + SD of 3 independent experiments.

prove that MRP2 is indeed solely dependent on stress fibers. Several options can be imagined as to the link between actin and MRP2 (Fig. 6). Actin could indirectly stabilize MRP2 by stabilizing the lipid rafts in which MRP2 resides (Fig. 6, option 3). Alternatively, actin could be directly linked to MRP2 in lipid rafts, or via a linker protein, such as one of the ERM proteins (Fig. 6, option 4). Human MRP2 is known to be able to link to actin via radixin through the carboxy-terminal cytoplasmic domain of MRP2, which was discovered in Rdx-/- mice (33). Radixin deficiency in these mice results in loss of MRP2 from bile canalicular membranes, which leads to conjugated hyperbilirubinemia, a phenotype very similar to that of Dubin-Johnson syndrome characterized by mutations in ABCC2, encoding MRP2. Thus, the ERM protein radixin in liver cells is essential for the correct localization and the conjugated bilirubin secretion function of MRP2. The concept of radixin-mediated canalicular localization of MRP2 was later confirmed in human cholestatic liver disease patients (34). Recently, this paradigm was extended to apical membrane localization of MRP2 in Caco-2 intestinal cells. Stable knockdown of radixin or ezrin using siRNA resulted in loss of MRP2 from the cell surface, while MRP2 and actin were detected in immunoprecipitates of cell lysates using anti-radixin or anti-ezrin antibodies (12). These results indicate that unlike the situation in hepatocytes, both ezrin and radixin are independently required for proper (apical membrane) localization of MRP2 in Caco-2 intestinal cells. Raft association of MRP2, actin and ERM proteins were not analyzed in these studies (12-14). In our study we observed that in concert with MRP2 also radixin shifted out of lipid rafts upon latrunculin B treatment. Therefore, our study is consistent with a role for radixin as intermediate between actin and MRP2 and for the first time emphasizes the involvement of lipid rafts as the platform where this interaction occurs. This study and our previous work (2) accumulate evidence for the notion that ABC transporters are more active when they are localized in actin-stabilized lipid rafts. Shifting out of lipid rafts consistently results in loss of activity of ABC transporters (Chapter 2, Fig. 2, option 3 or 4), while they remain fully active when their localization in lipid rafts does not depend on actin (Chapter 2, Fig. 6, option 2). Moreover, combining results of present and previous work shows that actin dependence is not specific for certain MRPs, but rather occurs in a cell type dependent manner. We have previously shown that localization and

function of MRP1 are dependent on cortical actin in Neuro-2a and BHK-MRP1 cells (paper Ina). Here we show that MRP1 is completely insensitive to actin in MDCKII cells. We speculate that MRP1 is linked to cortical actin in Neuro-2a and BHK-MRP1 cells according to model 3 (Chapter 2, Fig. 6), while in MDCKII cells it resides in lipid rafts according to model 2. MRP2 in MDCKII cells is speculated to be linked to the actin stress fibers via radixin according to model 4 (Chapter 2, Fig. 6).

It is important to mention, that in the case of MRP3 in the NOC/LAT condition, partial internalization of the transporter was observed with confocal microscopy, however, without any loss of efflux activity. This is consistent with previous results on MRP1 in Neuro-2a and BHK-MRP1 cells, which was also shown to internalize, while loss of its efflux activity was shown not to be related to this internalization (2).

In conclusion, this paper shows for the first time that various MRPs behave differently in terms of dependence on actin for their efflux activity. MRP1, MRP3 and MRP5 were – to a different extent – localized in lipid rafts that were not stabilized by actin, while MRP2 was in actin-stabilized lipid rafts, and likely connected to stress fibers via radixin. Upon actin disruption, both MRP2 and radixin shifted out of lipid rafts with a decrease of MRP2 function as result.

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Chapter 6

Pgp function is not susceptible to actin disruption

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Revised version resubmitted

Abstract

We have previously shown that MRP1 function and its localization in lipid rafts depend on cortical actin (Hummel I, Klappe K, Ercan C, Kok J.W. (2011) Mol. Pharm. 79.229-40). Here we show that Pgp function as an efflux pump did not depend on actin in the Pgp over expressing murine National Institutes of Health (NIH) 3T3 MDR1 G185 cell line as well as in human SK-N-FI cells, which endogenously express Pgp. Confocal laser scanning and electron microscopy revealed that both latrunculin B and cytochalasin D caused severe changes in cell and membrane morphology as well as changes in cellular distribution of Pgp. Nevertheless, the cell surface pool of Pgp remained similar to control conditions. Pgp in NIH 3T3 MDR1 G185 cells is partly localized in detergent-free lipid rafts in two different density gradient regions, which are both enriched in cholesterol and sphingolipids. Disruption of actin by cytochalasin D or latrunculin B did not change the density gradient distribution of Pgp. Our data show the first time that Pgp function as an efflux pump does not depend on (cortical) actin, which can be explained by stability of both the cell surface and the lipid raft pool of Pgp under conditions of actin disruption.

Introduction

The ABC transporter protein Pgp, which prevents intracellular drug accumulation, is known for its involvement in multidrug resistance of tumor cells. This transporter depends on its direct lipid environment for optimal functioning, having a higher affinity for its substrates when the surrounding lipids are in liquid-ordered phase rather than in the liquid-disordered phase (1-3). This is an important characteristic of lipid rafts (4,5) and Pgp has indeed been associated with lipid rafts. Lavie et al. (6) have shown for the first time the association of Pgp with lipid rafts (caveolae) in intact cells using Triton X-100 to isolate detergent-resistant membranes. In 2780AD human ovarian tumor cells, which lack caveolae, Pgp was located in DRMs defined by their insolubility in the detergent Lubrol WX (7). Pgp in the MDR Chinese hamster ovary cell line CHRB30 was localized in intermediate density domains isolated using Brij-96. These domains were shown to be distinct from caveolae as well as classic lipid rafts, as indicated by the differential gradient distribution of Pgp and the Src-family kinase Yes kinase (8). The mutual relationships between Pgp and lipid rafts have been elegantly reviewed (9).

Lipid rafts are often considered to be highly dynamic entities, but the actin cytoskeleton appears to have the potential to stabilize them (10). Interestingly, it has been inferred that Pgp is partially linked to the actin cytoskeleton (11). Moreover, Pgp-actin association through ezrin, radixin and moesin (ERM family proteins) has been shown in a MDR variant of the human T-lymphoblastoma cell line CEM-VBL100 (12). Recently we have shown that MRP1 is dependent on cortical actin for its efflux function. Moreover, concomitant with loss of function, MRP1 shifted out of lipid raft density gradient fractions (13).

In view of the observations on Pgp-actin interactions, it is important to note there are no studies which directly assess the effect of actin modulation on both Pgp-mediated efflux and its lipid raft association. Therefore, for the first time we investigated the impact of actin on both the membrane domain localization of Pgp and its efflux function, using confocal as well as electron microscopy, detergent-free lipid raft analysis and efflux assays. We conclude that actin disruption markedly changed cell morphology, including that of the

plasma membrane. However, in spite of changes in distribution of Pgp, there was no effect on Pgp-mediated efflux. Pgp was partly localized in lipid rafts and did not shift out of these lipid rafts upon actin disruption in NIH 3T3 MDR1 G185 cells. In accordance, Pgp expression on the cell surface was not changed. Pgp thus remains in its normal membrane environment and therefore remains active.

Materials and methods

Materials

All cell culture plastic was from Costar (Cambridge, MA, USA) and liquids from Gibco (Invitrogen, Paisley, UK). Fetal calf serum was from Bodinco (Alkmaar, The Netherlands). Latrunculin B, cytochalasin D, nocodazole, R123, the monoclonal anti-β-actin antibody and the monoclonal anti-Pgp antibody Clone F4 and calcein-AM were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 488-conjugated phalloidin and Alexa Fluor-conjugated secondary antibodies were obtained from Molecular probes (Eugene, OR, USA). The monoclonal anti-Pgp antibody MRK16 was from Abnova (Taipei City, Taiwan). The polyclonal anti-caveolin-1 antibody and the monoclonal anti-Rho-GDI antibody were from Transduction Laboratories (Lexington, KY, USA). The polyclonal anti-c-Src antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SK-N-FI cells were from ATCC (Wesel, Germany). OptiPrep was from Axis-Shield PoC AS (Dundee, Scotland).

Cell culture and incubation conditions

The NIH 3T3 mouse fibroblast cell line and its human MDR1-transfected counterpart (NIH 3T3 MDR1 G185) were kindly provided by Michael Gottesman, NIH, Bethesda, MD, USA (14). These cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate, under standard incubator conditions (humidified atmosphere, 5% CO_2, 37 °C). The NIH 3T3 MDR1 G185 cells were kept under selective pressure by growing them in the presence of 60 ng/ml colchicine. The cells were

trypsinized 1 day prior to the experiments and maintained without colchicine until use. The human SK-N-FI cell line with endogenous Pgp expression was cultured in the same medium, but supplemented with non-essential amino acids according to the manufacturer's suggestion. These cells were never treated with colchicine.

In order to disrupt the actin cytoskeleton in NIH 3T3 MDR1 G185 cells, cells were treated with cytochalasin D (10 µg/ml) or latrunculin B (0.3 or 10 µM) for 45 minutes. In some cases, cells were preincubated with nocodazole (10 µM) for 45 minutes to disrupt microtubules, followed by treatment with both nocodazole and latrunculin B for 45 minutes (13). SK-N-FI cells were treated similarly; however, 0.1 µg/ml cytochalasin D and 0.2 µM latrunculin B were used. Optimal concentrations of inhibitors were established based on efficacy of actin disruption and survival of the cells, since cell lines display different sensitivities towards these inhibitors. All incubations with cytoskeleton modulators and all control experiments without these modulators were performed in medium with serum.

Isolation of detergent-free lipid rafts

NIH 3T3 MDR1 G185 cells were cultured to confluence in 162 cm^2 flasks and treated with cytoskeleton modulators as mentioned above. Detergent-free lipid rafts were isolated as described (15,16).

Immunoblot analysis

Protein from equal volumes of the gradient fractions was trichloroacetic acid (TCA)-precipitated and resuspended in sample buffer (5% SDS, 5% β-mercaptoethanol, 0.125 M Tris-HCL, pH 6.8, 40% glycerol). The samples were processed for Western blot as described (16), using primary antibody against Pgp (1:2000), Src (1:2000), Rho-GDI (1:2000) or β-actin (1:2000).

Analysis of cholesterol, sphingolipid and protein content of OptiPrep gradient fractions

Lipids were extracted from pooled OptiPrep gradient fractions (17). The cholesterol concentration was determined spectrophotometrically by a cholesterol oxidase/peroxidase assay (18). Sphingolipids were extracted from pooled OptiPrep gradient fractions and

analyzed by liquid chromatography-electrospray ionization tandem mass spectrometry, as described (16). The amounts of individual sphingolipid species were added to obtain the total sphingolipid pool. Protein content of pooled OptiPrep gradient fractions was determined as described by Smith et al. (19).

Detection of Pgp-mediated rhodamine 123 efflux

Cells were cultured to confluence in 25 cm^2 flasks and treated with cytoskeleton modulators as mentioned above. Cells were harvested by trypsinization, washed with Hank's balanced salt solution and incubated with the Pgp substrate R123 (10 µM in HBSS) at 10 °C for 60 minutes. To rule out a substrate concentration dependent effects, all experiments were performed at 3 times higher (30 µM) and 3 times lower concentrations (3.3 µM) of R123 as well. After the R123 incubation cells were washed with ice-cold HBSS. Subsequently, the cells were incubated at 37 °C in the presence or absence of the Pgp inhibitor cyclosporin A (10 µM) during various time intervals. The efflux of the fluorescent substrate was stopped by mixing the cells with ice-cold HBSS containing CSA (10 µM final concentration) and keeping them on ice. The remaining cell-associated fluorescence was determined by flow cytometric analysis using an Elite flow cytometer (Beckman Coulter, Miami, FL).

Detection of Pgp-mediated calcein efflux

Extrusion of calcein-acetoxymethyl ester was measured as described earlier (20) with the following changes. Cells were trypsinized, washed, resuspended in HBSS ($7x10^5$ cells/ml) and plated in 96 well solid white polystyrene microplates (100 µl/well). For Pgp inhibition, cells were incubated with CSA (10 µM final concentration). Then 100 µl/well calcein-AM mix (0.07 mg/ml bovine serum albumin (BSA) in HBSS with 0.5 µM or 0.16 µM or 1.5 µM calcein-AM) or alternatively calcein-AM mix supplemented with 10 µM CSA was added and fluorescence was measured in 30 second cycles on a LS55 luminescence spectrometer (Perkin Elmer, Waltham, MA, USA).

The calculated Multidrug Activity Factor is defined as:

$MAF=[(R_{inh} - R_{con})/R_{inh}]x100$.

R_{inh} is the slope of the calcein accumulation curve of cells incubated with 10 µM CSA. R_{con} is the slope of the calcein accumulation curve of cells without CSA. The difference between R_{inh} and R_{con} depends on the number and activity of the Pgp transporter molecules present in the cell membranes. Accordingly, a high MAF value corresponds to a high number and/or activity of Pgp molecules in the membrane (20).

Detection of the Pgp cell surface pool

We used a modified version of the method described by Druley T.E. et.al. (21). Cells were cultured in 25 cm^2 flasks, treated with cytoskeleton modulators as mentioned above and harvested by trypsinization. The cells were kept at 4 °C to prevent recovery, washed twice with ice-cold PBS containing 1% BSA (PBS-BSA) and centrifuged at 4 °C. $2.5x10^5$ NIH 3T3 MDR1 G185 cells or $2x10^5$ SK-N-FI cells were suspended in 50 µl of ice-cold PBS-BSA containing 40 µg/ml of human Pgp-specific MRK16 monoclonal antibody and incubated for 30 min at room temperature together with the adequate cytoskeleton modulators. Primary antibody reactions were stopped with 1 ml of ice-cold PBS and cells were centrifuged (4.5 min, 4 °C, 1400 rpm) followed by washing once with cold PBS-BSA. Pellets were resuspended in 50 µl cold PBS-BSA containing goat anti-mouse Alexa Fluor 488-conjugated secondary antibody (600x or 500x diluted for NIH 3T3 MDR1 G185 and SK-N-FI cells, respectively). The reaction mixtures were incubated at 4 °C for 40 min and the reactions were stopped by dilution in 1 ml of ice-cold PBS followed by centrifugation (4.5 min, 4 °C, 1400 rpm). Prior to FACS analysis each pellet was resuspended in 250 µl of PBS-BSA. Fluorescence was determined by flow cytometric analysis as described above. A mouse IgG2a isotype primary antibody was used (5 µl in 50 µl PBS-BSA) as negative control according to the manufacturer's suggestions.

Confocal laser scanning fluorescence microscopy

Cells were grown to 40-60 % confluence on glass cover slips in 12 well plates and treated with cytoskeleton modulators as mentioned above. Cells were processed for confocal microscopy as described (13). Cells were stained with antibody against Pgp (antibody Clone F4; 1:150) and appropriate Alexa Fluor 546-conjugated secondary antibody. In most

experiments, cells were co-stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin. Analysis of the samples was performed using a TCS Leica SP2 AOBS Confocal Laser Scanner Microscope (Leica, Heidelberg, Germany), equipped with a HCX PL APO 63x 1.4 oil CS objective in combination with Leica Confocal Software. The system was operated in the sequential mode in case of double labeled samples. Images were processed using Adobe Photoshop CS3.

Electron microscopy

NIH 3T3 MDR1 G185 cells were grown to 70 % confluence in 12 well plates and treated with cytoskeleton modulators as mentioned above. Cells were then fixed in 2 % paraformaldehyde (PFA), 0.01 % glutaraldehyde, pH 7.4, 1 hour on ice. Cells were washed with 0.1 M cacodylate buffer (pH 7.4) for 15 minutes, then osmicated in 1% OsO_4, 1.5% potassium hexacyanoferrate in cacodylate buffer for 30 minutes, washed with water, dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections (50-70 nm) were cut on a Leica Ultracut E, counterstained with uranyl acetate and lead citrate, and examined with a Philips CM 100 transmission electron microscope.

Results

Validation of the model cell lines

This study aims to determine the effect of actin on Pgp localization and function and relies on the use of model cell lines that express active Pgp. Western blotting confirmed the presence of Pgp in NIH 3T3 MDR1 G185 (Fig. 1A) and SK-N-FI cells (Fig. 1B), while it was not detected in parental NIH 3T3 cells (Fig. 1A). Due to the lower expression of Pgp in SK-N-FI cells compared to NIH 3T3 MDR1 G185 cells, Pgp was only detectable in the former after performing membrane isolation, thus increasing the oncentration of Pgp relative to total protein in the sample. In contrast to parental NIH 3T3 cells, NIH 3T3 MDR1 G185 cells effectively effluxed the Pgp substrate R123 (Fig. 1C). The efflux from NIH 3T3 MDR1 G185 cells was strongly inhibited by CSA, which is consistent with the notion

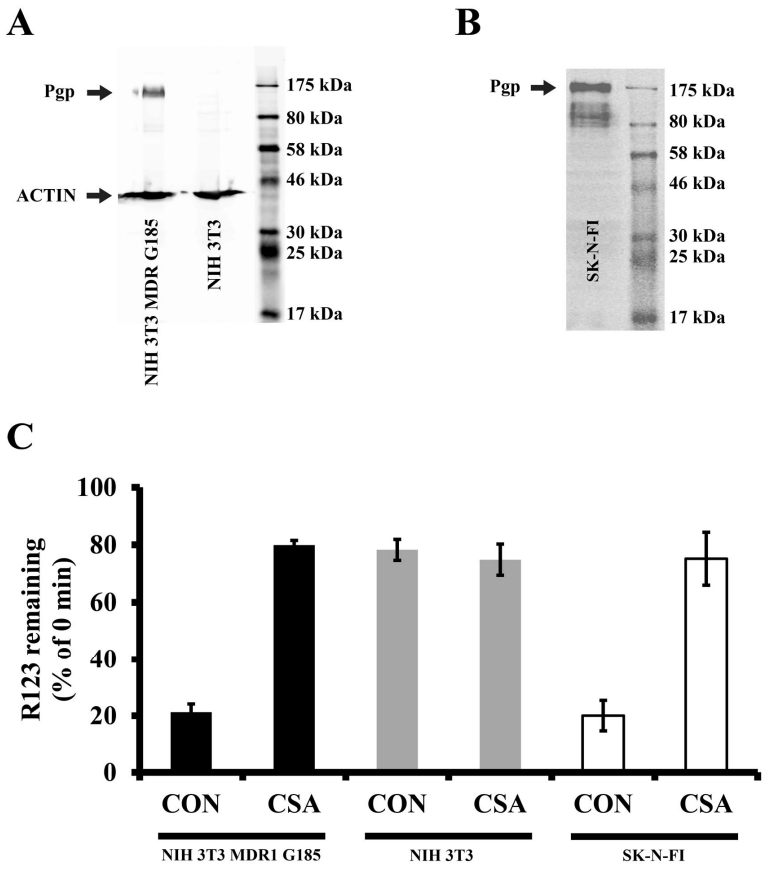

Figure 1. Validation of the Pgp model cell lines NIH 3T3 MDR1 G185 and SK-N-FI
(**A-B**) Whole cells (NIH 3T3 MDR1 G185 and parental NIH 3T3) or isolated membranes (SK-N-FI) were processed for Western blotting and Pgp was detected using the anti-Pgp antibody Clone F4 in NIH 3T3 MDR1 G185 (A) and SK-N-FI cells (B), but not in parental NIH 3T3 cells (A).
C) Cells were loaded with the Pgp substrate R123 and efflux was allowed to proceed for various time intervals. Efflux in NIH 3T3 MDR1 G185 as well as SK-N-FI cells was effectively inhibited by CSA and the residual loss of R123 in case of NIH 3T3 MDR1 G185 cells is equal to that in parental NIH 3T3 cells, with or without CSA. Data represent the mean +/- SD of 3 independent experiments.

that Pgp was the active transporter (Fig. 1C, black columns). After CSA treatment, the remaining R123 level is very similar to that in NIH 3T3 cells, which do not express Pgp (Fig. 1C, grey columns). Also SK-N-FI cells effectively effluxed the Pgp substrate R123, and this was strongly inhibited by CSA (Fig. 1C, white columns). In conclusion, NIH 3T3

MDR1 G185 and SK-N-FI cells express active Pgp on the cell surface. Having validated the model cell line, we continue our study on Pgp localization and function.

Actin disruption results in changes in cell and membrane morphology

The actin cytoskeleton (Fig. 2A) is composed of stress fibers (asterisk) and cortical actin (arrowhead) which is localized close to the plasma membrane. We first established the efficacy of two modulators of the actin cytoskeleton, i.e cytochalasin D and latrunculin B, to disrupt stress fibers and/or cortical actin. In case of NIH 3T3 MDR1 G185 cells (Fig. 2D), upon treatment with 10 µg/ml cytochalasin D stress fibers were disrupted, but some continuous cortical actin was still present (arrowhead), while actin accumulated in the cytoplasm and as patches (arrow) in distinct regions near the plasma membrane. The use of a low concentration of latrunculin B (0.3 µM) resulted in stress fiber disruption while cortical actin was still localized cortically, although it was no longer continuous (Fig. 2G; arrowhead). Treatment with 10 µM latrunculin B resulted in loss of both stress fibers and cortical actin, while actin accumulations were found close to the plasma membrane (Fig. 2L; arrowhead). In 10 µM latrunculin B-treated cells after pretreatment with nocodazole (Fig. 2P), linear or punctuate cortical actin was observed (arrowhead), but more often irregular shaped vesicle formations and actin accumulations outside the cell (arrow). Taken together, results show that stress fibers are disrupted after both 10 µM latrunculin B and 10 µg/ml cytochalasin D treatments. However, cortical actin is affected differently by the two modulators at these concentrations, rendering them as valuable tools for studying the role of cortical actin in function and localization of Pgp. Cell morphology is highly affected with both modulators, resulting in cell rounding.

In case of SK-N-FI cells (Fig. 3A) cortical actin was readily visible (arrowhead), while stress fibers appeared less well organized compared to NIH 3T3 MDR1 G185 cells (Fig. 2A). These cells were much more sensitive to the actin modulators and did not survive the concentrations as used in NIH 3T3 MDR1 G185 cells (data not shown). Therefore the concentrations were again optimized. Treatment with 0.1 µg/ml cytochalasin D resulted in actin accumulations throughout the cell, while cortical actin was still

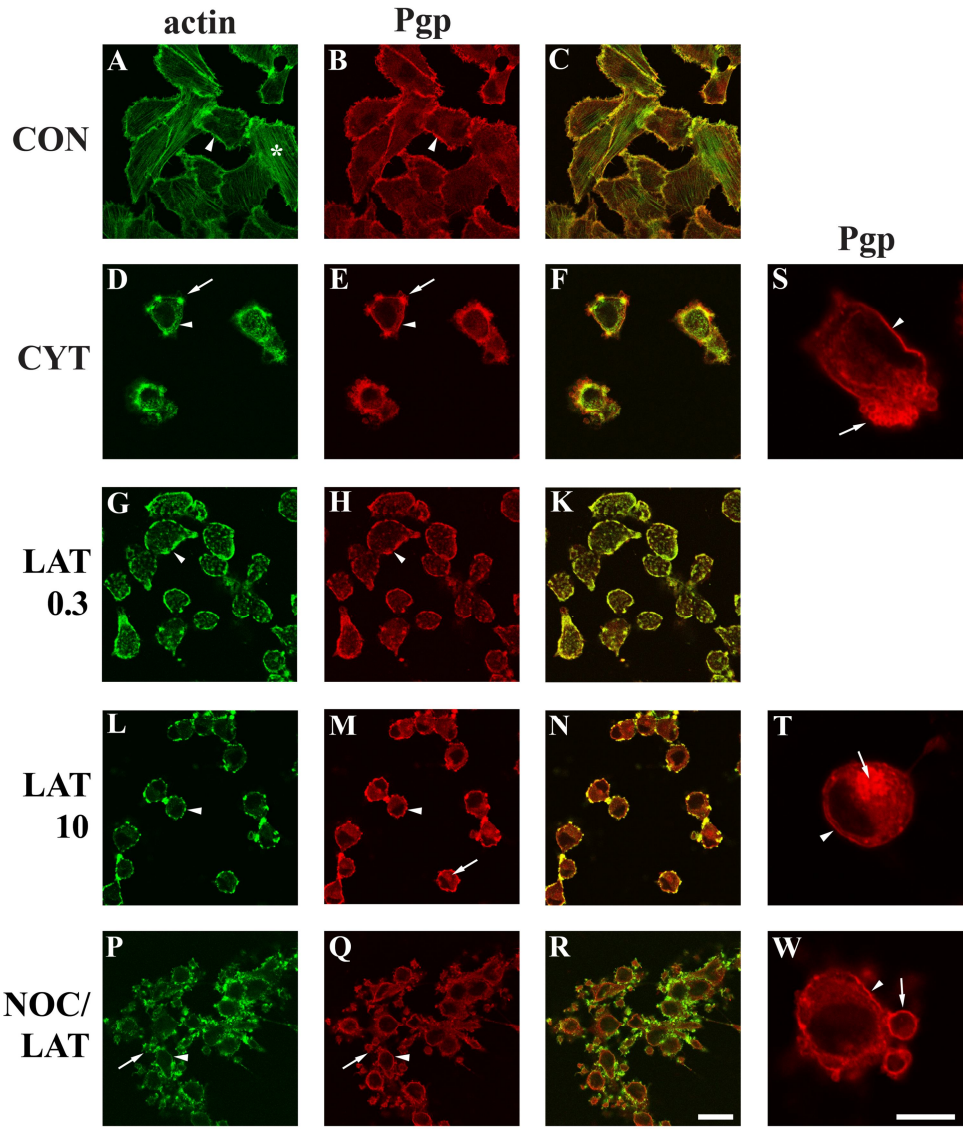

Figure 2. Disruption of actin and altered distribution of Pgp after cytochalasin D and latrunculin B treatment in NIH 3T3 MDR1 G185 cells

NIH 3T3 MDR1 G185 cells were untreated (CON; A-C), or treated with 10 µg/ml cytochalasin D (CYT; D-F and S), with 0.3 µM latrunculin B (LAT 0.3; G-K), with 10 µM latrunculin B (LAT 10; L-N and T), or with 10 µM nocodazole followed by 10 µM nocodazole + 10 µM latrunculin B (NOC/LAT; P-R and W). Cells were co-stained for actin (Alexa Fluor 488-conjugated phalloidin) and Pgp (anti-Pgp antibody Clone F4). C,F,K,N,R are overlay images. Arrowheads indicate cortical actin, the expected location of cortical actin or alternatively Pgp in the plasma membrane. Arrows indicate a patch of actin or Pgp. The asterisk indicates stress fibers. Bars, 20 µm, exept for S,T,W: 10 µm.

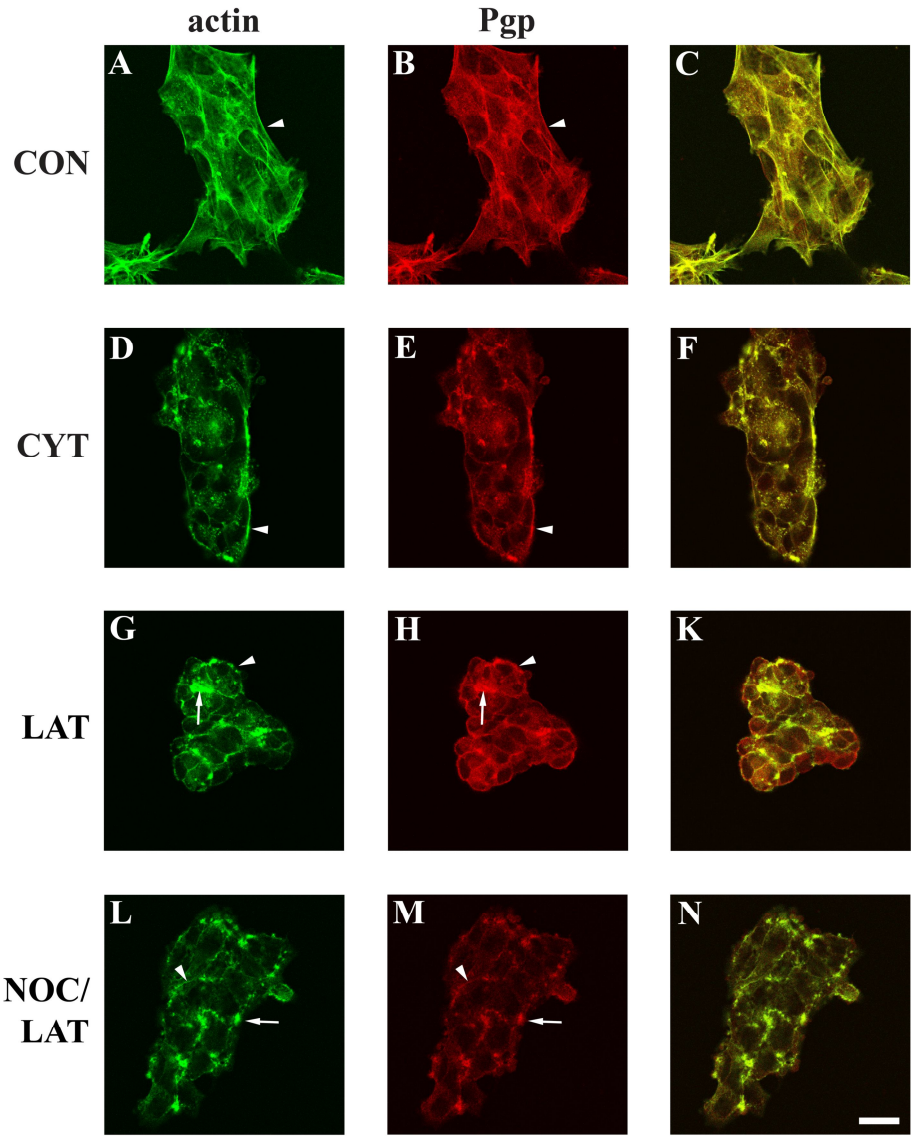

Figure 3. Disruption of actin and altered distribution of Pgp after cytochalasin D and latrunculin B treatment in SK-N-FI cells
SK-N-FI cells were untreated (CON; A-C), or treated with 0.1 µg/ml cytochalasin D (CYT; D-F), with 0.2 µM latrunculin B (LAT; G-K), or with 10 µM nocodazole followed by 10 µM nocodazole + 0.2 µM latrunculin B (NOC/LAT; L-N). Cells were co-stained for actin (Alexa Fluor 488-conjugated phalloidin) and Pgp (anti-Pgp antibody Clone F4). C,F,K,N are the overlay images. Arrowheads indicate cortical actin or the expected location of cortical actin or alternatively Pgp in the plasma membrane. Arrows indicate a patch of actin or Pgp. The asterisk indicates stress fibers. Bars, 20 µm.

observed (Fig. 3D; arrowhead). Upon 0.2 µM latrunculin B treatment (Fig. 3G) larger actin accumulations were found (arrow) and cortical actin was disrupted yielding an irregular and discontinuous pattern near the plasma membrane (arrowhead). Latrunculin B treatment caused cell rounding, as was observed with NIH 3T3 MDR1 G185 cells. In 0.2 µM latrunculin B-treated cells after pretreatment with nocodazole (Fig. 3L), linear or punctuate cortical actin was observed (arrowhead), but more often irregular shaped actin accumulations near the cell surface (arrow), reminiscent of the pattern observed in NIH 3T3 MDR1 G185 cells (Fig. 2P).

To characterize the morphological changes of the NIH 3T3 MDR1 G185 cells in more detail, accompanying the cytoskeletal perturbations induced by the actin modulators, as clearly visible by light microscopy (Fig. 2), we performed electron microscopy. Control cells displayed a leading edge (Fig. 4A) with filopodia (Fig. 4B). Upon treatment with 10 µg/ml cytochalasin D, the most apparent change was the appearance of large membrane-enclosed structures outside the cells, often in a distinct region near the plasma membrane (Fig. 4, C and D). This is reminiscent of the actin accumulation observed by confocal microscopy (Fig. 2D). The use of a low concentration of latrunculin B (0.3 µM) resulted in cell rounding (Fig. 4E), as well as partial loss of filopodia (Fig. 4F). A high concentration of latrunculin B (10 µM) caused rounding of the cells and clustering of organelles (Fig. 4G) while filopodia were absent from the plasma membrane (Fig. 4H). Large vacuoles were observed beneath the plasma membrane (Fig. 4H). Finally, when latrunculin B treatment (10 µM) was preceded by treatment with nocodazole to disrupt the microtubules, cell rounding was observed but no clustering of organelles (Fig. 4K). Very large membrane-enclosed structures appeared outside the cells (Fig. 4K). Large vacuoles beneath the plasma membrane were apparent and the plasma membranes itself displayed deformations (Fig. 4L).

Taken together, cytochalasin D, latrunculin B and the combination nocodazole/ latrunculin B caused substantial alterations in the morphology and integrity of the cell surface. Whether perturbations of the actin cytoskeleton and ensuing changes in membrane surface integrity might affect the distribution and function of membrane localized Pgp, was examined next.

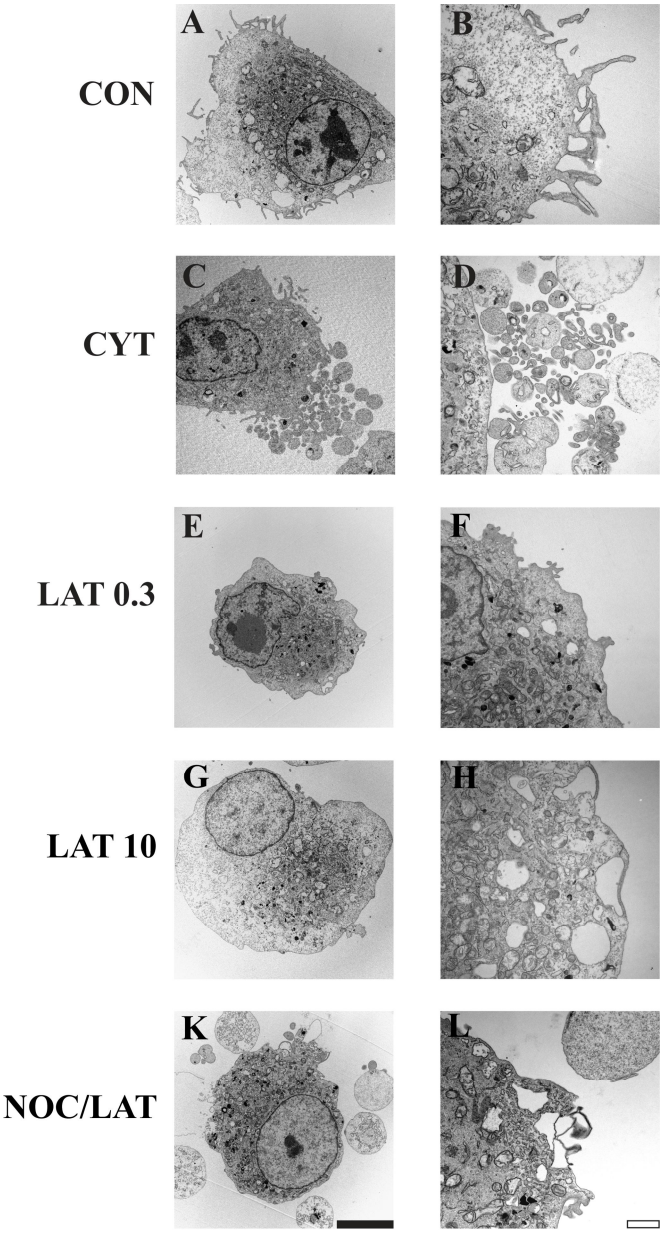

Figure 4. Disruption of actin results in changes in cell and plasma membrane morphology in NIH 3T3 MDR1 G185 cells

Cells were treated as described in Fig. 2 and processed for electron microscopy. Left images were used for assessment of cell morphology, right images for assessment of membrane morphology. Closed bar 10 µm; open bar 2 µm.

Actin disruption results in changes in Pgp localization

In control NIH 3T3 MDR1 G185 cells, Pgp was uniformly distributed over the plasma membrane (Fig. 2B; arrowhead). When cells were treated with cytochalasin D, Pgp accumulated in patches in a distinct region of the plasma membrane (Fig. 2E; arrow), similar to the result obtained with actin staining (Fig. 2D). Detailed imaging showed that the large extracellular membrane-enclosed structures, reminiscent of those observed with electron microscopy (Fig. 4, C and D), were positive for Pgp staining (Fig. 2S; arrow), as well as the plasma membrane (arrowhead). In contrast to 0.3 µM latrunculin B treatment (Fig. 2H), treatment with 10 µM latrunculin B resulted in an Pgp staining pattern suggestive of partial internalization of Pgp (Fig. 2M; arrow). Indeed, detailed imaging showed intracellular staining of Pgp (Fig. 2T; arrow), distinct from the plasma membrane staining (arrowhead). When latrunculin B treated cells were pre- and co-treated with nocodazole, prominent Pgp localization in the plasma membrane was apparent (Fig. 2Q,W; arrowhead). Moreover, the numerous and severe plasma membrane deformations and extracellular membrane-enclosed structures arising at these conditions, as revealed by electron microscopy (Fig. 4L), appeared to contain Pgp (Fig. 2W; arrow).

The Pgp staining pattern in SK-N-FI cells closely resembled that of actin in all conditions (Fig. 3B,E,H,M). Only in the case of latrunculin B (Fig. 3G-K), the staining patterns slightly differed between Pgp and actin, with more intracellular staining of Pgp. In contrast to control cells, Pgp displayed large accumulations in latrunculin B treated cells (Fig. 3H; arrow) as well as those cells pretreated with nocodazole (Fig. 3M; arrow).

Taken together, our results from confocal and electron microscopy showed that actin disruption resulted in changes in both cell and plasma membrane morphology as well as Pgp distribution, including internal and external localizations of the transporter.

Actin disruption does not affect Pgp-mediated efflux function

We measured the kinetics of Pgp-mediated efflux of R123 in the absence or presence of actin modulators in both NIH 3T3 MDR1 G185 and SK-N-FI cells. The cells were first incubated with actin modulators, followed by analysis of the efflux of R123. No differences were observed between all conditions, including cytochalasin D or

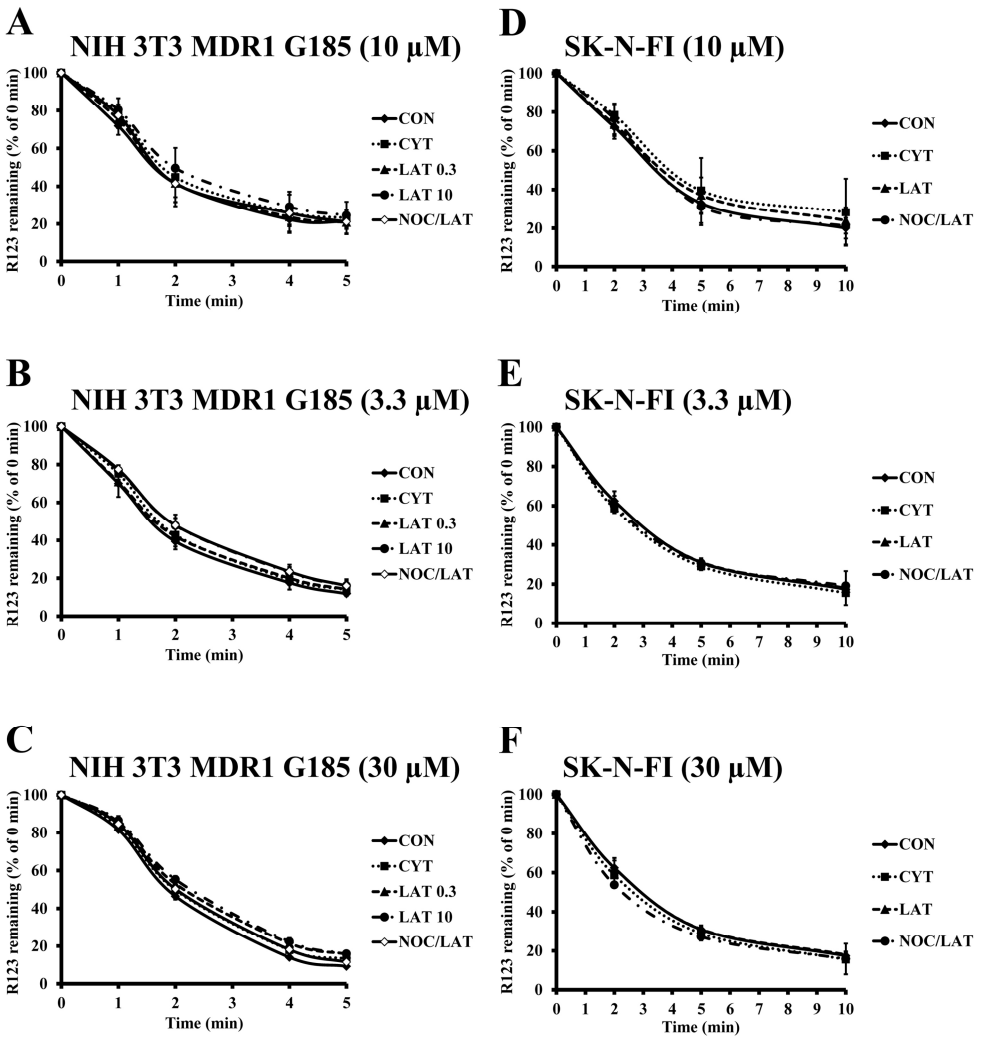

Figure 5. Disruption of actin does not result in changes in efflux function of Pgp in NIH 3T3 MDR1 G185 cells and SK-N-FI cells
Cells were treated as described in Fig. 2 or 3. **A-C**) Efflux kinetics of R123 under these conditions in NIH 3T3 MDR1 G185 cells. **D-F**) Efflux kinetics of R123 under these conditions in SK-N-FI cells. The R123 concentration was 10 µM (A,D) and this was lowered to 3.3 µM (B,E) or increased to 30 µM (C,F). Values are presented as % of 0 minutes +/- SD (n=3).

latrunculin B treatment (Fig. 5A and D). This leads to the conclusion that Pgp-mediated efflux function remained normal, in spite of changes in the distribution of the ABC transporter. Although the assay is reliable and the results are straightforward, we decided to

substantiate these results in two ways: 1. The experiments were carried out with a 3 times lower (Fig. 5B and E), and also with a 3 times higher (Fig. 5C and F) concentration of the substrate R123, in order to exclude the possibility that an effect of actin modulators becomes apparent at another substrate concentration. 2. Pgp-mediated efflux was measured with another assay, based on a different substrate, calcein-AM (Fig. 6).

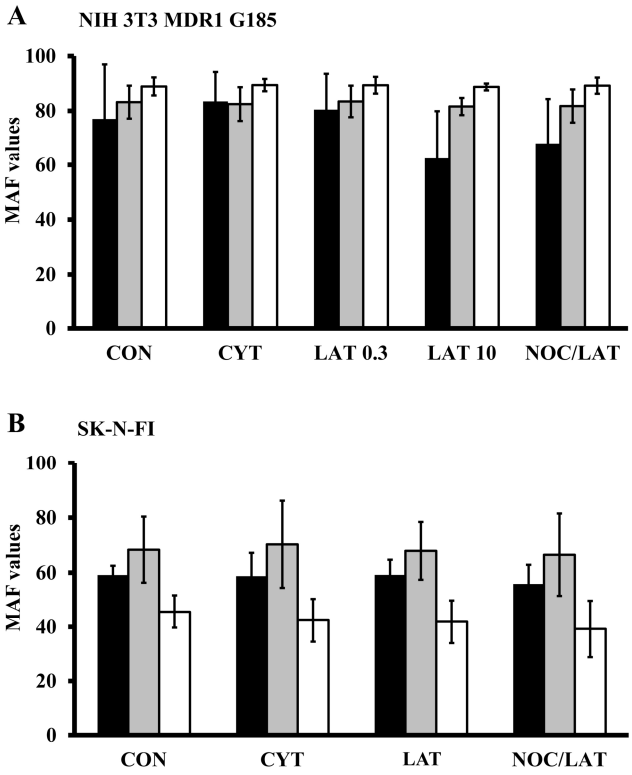

Figure 6. Disruption of actin does not result in changes in efflux function of Pgp in NIH 3T3 MDR1 G185 cells and SK-N-FI cells
Cells were treated as described in Fig. 2 or 3. Efflux of calcein-AM under these conditions in NIH 3T3 MDR1 G185 cells **A**), or SK-N-FI cells **B**). The standard calcein concentration was 10 (black bars) µM and this was lowered 3x to 3.3 µM (gray bars) or increased 3x to 30 µM (white bars). Values are presented as Multidrug Activity Factor ± SD (n=4). There were no significant differences between cytoskeleton conditions, as determined by the one-way ANOVA test ($P > 0.05$).

This was performed in both cell lines and also with three different substrate concentrations. Also with the calcein efflux assay no differences were observed between all conditions,

including cytochalasin D and latrunculin B treatment, for both NIH 3T3 MDR1 G185 cells (Fig. 6A) and SK-N-FI cells (Fig. 6B). In conclusion, under conditions were cell and membrane morphology have changed, Pgp-mediated efflux function remained normal, in spite of changes in the subcellular distribution of the ABC transporter.

Actin disruption does not affect Pgp localization in lipid rafts

We used a detergent-free method for the isolation of lipid rafts from NIH 3T3 MDR1 G185 cells and first characterized the gradient fractions in terms of cholesterol and sphingolipid enrichment. Fractions 1-2 were strongly and significantly enriched in both cholesterol (Fig. 7A) and sphingolipids (Fig. 7B). To a slightly lesser extent this was also true for the fractions 3-4, which indicates that these fractions truly represent lipid rafts, but with a slightly higher buoyant density than those in fractions 1-2. In accordance with the lipid raft nature of the membranes in fraction 3-4, the lipid raft marker Src was mostly enriched in these density gradient fractions (Fig. 7C). As expected, the non-raft marker Rho-GDI was mostly found in the non-raft fractions 7-9 (Fig. 7D). Some Pgp was found in the lipid raft fractions 1-2, but the ABC transporter was more enriched in the lipid raft fractions 3-4, together with Src (Fig. 7E, compare to 7C). The largest pool of Pgp was not lipid raft associated, as it was found in fractions 7-9, together with Rho-GDI (Fig. 7E, compare to 7D). Upon actin disruption, under all conditions there was no shift of Pgp out of the lipid raft fractions 3-4. The amount of Pgp in the lipid raft fractions 1-2 showed a tendency to decrease with latrunculin B treatment, either with or without preincubation with nocodazole (Fig. 7E), but this was not significant ($P > 0.05$). In conclusion, in accordance with the absence of an effect of actin modulators on Pgp-mediated efflux, there was no effect on the extent of lipid raft association of Pgp in NIH 3T3 MDR1 G185 cells.

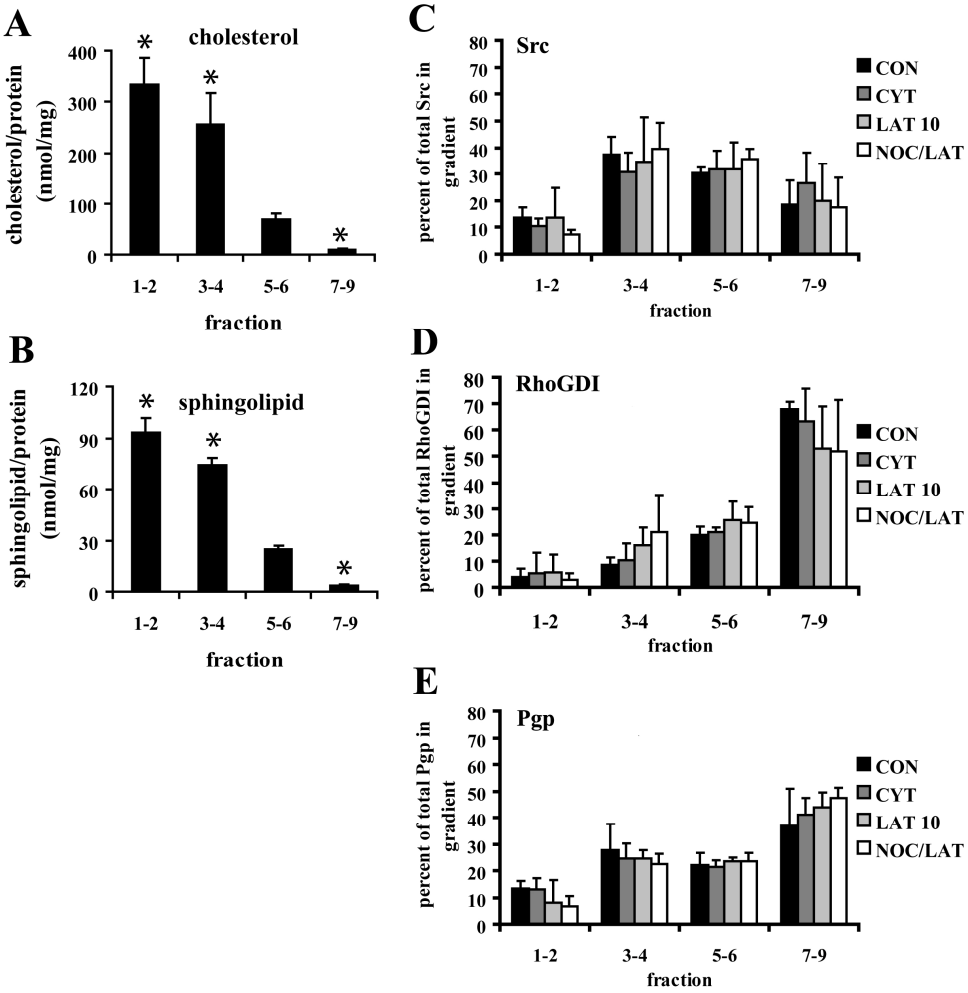

Figure 7. Disruption of actin does not result in loss of association of Pgp with cholesterol- and sphingolipid-enriched lipid raft fractions in NIH 3T3 MDR1 G185 cells

A,B) Lipid rafts were isolated from cells using a detergent-free method. In the pooled fractions, cholesterol (A) and sphingolipids (B) were measured, as well as protein content. The two lipid classes are expressed in nmols relative to protein content in mg of the pooled gradient fractions. Data represent the mean + SD of 3 independent experiments. *Values are significantly ($P < 0.05$) different from those of fractions 5-6 (non-lipid raft membrane fractions) as determined by Student's t-test.

C-E) Cells were treated as described in Fig. 2, processed for lipid raft analysis as described above (A,B) and Western blotting. Blots were stained for the lipid raft maker Src (C), the non-raft marker Rho-GDI (D) and Pgp using antibody Clone F4 (E) and quantified with the Odyssey protocol. The numbers indicate the percentage of a specific protein found in pooled gradient fractions, relative to the total of that protein in the entire gradient. Data represent the mean + SD of 3 independent experiments. There were no significant differences between cytoskeleton conditions, as determined by the one-way ANOVA test ($P > 0.05$).

Actin disruption does not affect the Pgp cell surface pool

Finally, we measured the pool of Pgp accessible from the outside of the cells to establish whether or not this pool had changed after actin modulation.

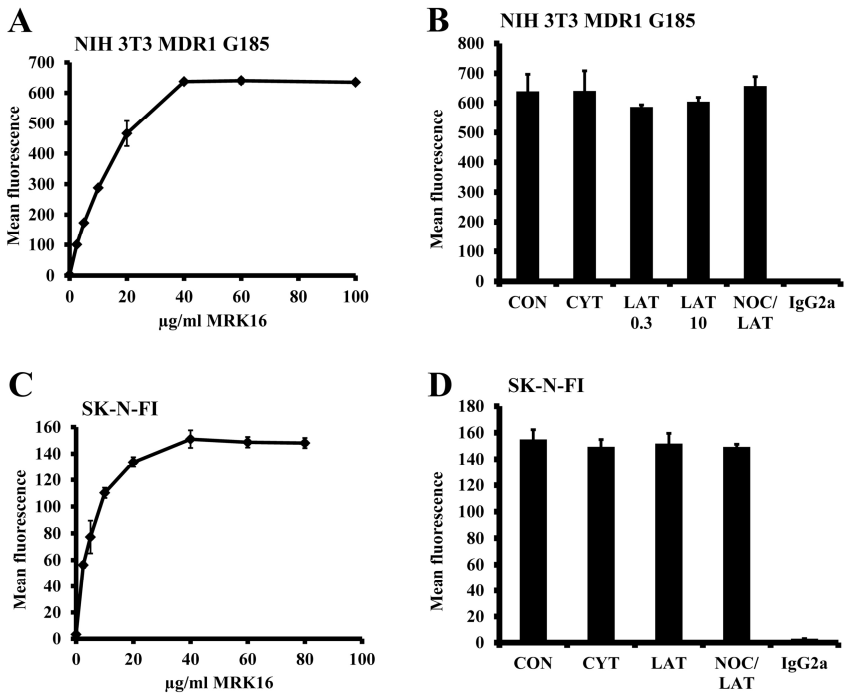

Figure 8. Disruption of actin does not results in reduced surface expression of Pgp in NIH 3T3 MDR1 G185 and SK-N-FI cells

Cells were treated as described and processed for flow cytometric analysis using the Pgp antibody MRK16, which recognizes an external epitope of Pgp and a secondary fluorescent antibody. **A,C**) Saturation curves were constructed for NIH 3T3 MDR1 G185 (A) and SK-N-FI (C) cells. **B,D**) All cytoskeleton conditions were tested for cell surface labeling of Pgp in NIH 3T3 MDR1 G185 (C) and SK-N-FI cells (D) using 40 µg/ml of MRK16. A mouse IgG2a isotype primary antibody was used as negative control. Data represent the mean + SD of 3 independent experiments. There were no significant differences between cytoskeleton conditions, as determined by the one-way ANOVA test ($P > 0.05$).

This represents the pool on the cell surface which is likely the only pool involved in efflux of Pgp substrates we measured using the R123 and calcein assays. To this end we performed flow cytometry analysis using the Pgp antibody MRK16, which recognizes an external epitope of Pgp, and a secondary fluorescent antibody. The assay was first optimized by constructing a saturation curve for the MRK16 antibody for both the NIH 3T3

MDR1 G185 (Fig. 8A) and SK-N-FI cell lines (Fig. 8C). Experiments with the actin modulators were then performed using the optimal MRK16 antibody concentration (40 µg/ml). There were no differences in the cell surface pool under all conditions, including cytochalasin D and latrunculin B treatment in both NIH 3T3 MDR1 G185 (Fig. 8B) and SK-N-FI cells (Fig. 8D). It was also apparent and consistent with the Western blot data that Pgp was much lower expressed in SK-N-FI cells compared to NIH 3T3 MDR1 G185 cells. In conclusion, in spite of drastic changes in plasma membrane morphology, i.e. membrane deformations and external membrane-enclosed structures, as well as changes in distribution of Pgp, the pool of Pgp expressed on the surface remained the same.

Discussion

In this study we have shown that Pgp is localized on the cell surface of both Pgp over expressing NIH 3T3 MDR1 G185 cells and SK-N-FI cells, which endogenously express Pgp. The expression of Pgp in SK-N-FI cells is much lower than that in NIH 3T3 MDR1 G185 cells, as shown by Western blot which requires prior membrane isolation in the case of SK-N-FI cells and by the surface labeling experiments. However, the efflux activity of the transporter can readily be measured in both cell lines. The main conclusion of this paper is that actin disruption does not affect the efflux function of Pgp in these two cell lines. Under conditions where cell and membrane morphology have changed, Pgp-mediated efflux function remained normal, in spite of changes in the subcellular distribution of the ABC transporter. It is important to establish this in two different cell lines, to support the notion that it is not a cell type dependent effect. Moreover, the NIH 3T3 MDR1 G185 cell line is a murine cell line subjected to force over expression of the human Pgp transporter and this cell line is under selective pressure of colchicine. One or both of these factors could have hampered an interaction of human Pgp with murine actin. Therefore we corroborated this result in the SK-N-FI cell line, to our knowledge the only cell line available with endogenous expression of the human Pgp in a human cell background and without any selective pressure by cytostatics.

In NIH 3T3 MDR1 G185 cells, Pgp is partly localized in lipid raft domains and partly non-lipid raft associated. The lipid raft domains were retrieved as fractions 3-4 as well as 1-2 by density gradient analysis after detergent-free raft isolation and they were characterized by enrichment of the typical raft lipids cholesterol and sphingolipids. Pgp, and also the lipid raft marker Src, were more abundantly associated with the lipid raft fractions 3-4, as compared to 1-2. These fractions were relatively poor in the non-lipid raft marker Rho-GDI. Both the extent of Pgp association with lipid rafts and the Pgp-mediated efflux function were unaffected by actin disruption. This is in contrast to results we recently obtained for MRP1 under similar conditions. MRP1 displayed reduced efflux activity after latrunculin B treatment concomitant with a shift out of lipid raft fractions (13). MRP1, however, was most abundant in the lipid raft fractions 1-2, and the shift occurred from these fractions. Like Pgp, also MRP1 did not shift from fractions 3-4 upon latrunculin B treatment (13). Therefore, we come to the conclusion that different types of membrane domains are isolated in fractions 1-2 versus 3-4. Both are enriched in typical raft lipids and lipid raft markers, but they differ concerning their sensitivity towards (cortical) actin disruption. MRP1 associated with the lipid raft fractions 1-2 is sensitive to (cortical) actin-disruption (13), while both MRP1 (13) and Pgp (current study) associated with the lipid raft fractions 3-4 are not sensitive. In accordance, the amount of Pgp in the lipid raft fractions 1-2 showed a tendency (not significant) to decrease with latrunculin B treatment. Confirmation of these results in the SK-N-FI cell line would be of use; however we could not detect Pgp in the fractions obtained after lipid raft isolation due to the low expression of Pgp in this cell line and the dilution of the protein inherent in this procedure. This could not be overcome by further pooling of gradient fractions or immunoprecipitation of Pgp from the gradient fractions.

Taken together, Pgp in NIH 3T3 MDR1 G185 cells appears to be partly localized in lipid rafts. 1) Upon (cortical) actin disruption, Pgp and Src do not shift out of lipid raft fractions, suggesting that these lipid rafts are stable independent of actin. Moreover, a significant part of Pgp is not lipid raft associated. Pgp thus remains in its normal membrane micro-environment after (cortical) actin disruption. 2) In addition, we have shown in both cell lines that the cell surface pool of Pgp does not change upon actin disruption in spite of

drastic changes in membrane morphology and redistribution of Pgp. Apparently, these two conditions allow normal efflux function of the ABC transporter. Our results obtained with nocodazole pre-treatment show that in addition to the actin network, microtubules are not needed to support Pgp-mediated efflux function in both cell lines and its localization in lipid rafts in NIH 3T3 MDR1 G185 cells. Moreover, under these conditions of double treatment (nocodazole and latrunculin B), the membrane deformations were quite severe and this shows that Pgp can function normally in a highly perturbed membrane. We conclude that the concept of a functional Pgp-actin linkage, as described by Luciani et al. (12), is not universal. Their study on Pgp-actin association through ezrin, radixin and moesin was performed in a radically different cell type, the human T-lymphoblastoma cell line CEM-VBL100. On the other hand, Bacso et al. (11) studied Pgp-actin linkage in NIH 3T3 MDR1 G185 cells. They concluded that there is a pool of Pgp, which is directly linked to actin. However, the evidence was indirect and the consequences of Pgp linkage to actin for Pgp-mediated efflux function were not investigated in this study. Therefore, although actin may be a promising target for strategies aimed at overcoming MDR through inhibition of ABC transporter function, this concept may highly depend on the cell type and the way the ABC transporter is organized in the membrane with respect to various types of lipid rafts and their variation in association with the actin network. Our results imply that (cortical) actin is not a universal target in combating Pgp-mediated drug resistance and that Pgp remains effective as an efflux pump in cells with drastic morphological changes and even severe plasma membrane perturbations.

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Chapter 7

Summary and discussion of the Thesis

Nederlandse samenvatting

Magyar nyelvű összefoglaló

Acknowledgements

About the author

List of publications

Summary

Cancer is one of the most serious diseases in the developed countries and is usually treated with chemotherapy, especially when it concerns a disseminated disease. If not all of the tumor cells are eradicated, reoccurrence of the tumor and associated multidrug resistance of the tumor cells is a huge problem for follow up therapy. Multidrug resistance is often caused by (over)expression of ABC transporters. These notions have since long drawn the attention of scientists as well as drug development companies to this important research field. Today we know that ABC transporters are multispanning membrane proteins with the ability to pump molecules with a large structural variety out of the tumor cell. ABC transporters form one of the largest protein families and are present in all known organisms from bacteria to humans. The presence of these proteins is actually one of our main defense mechanisms to protect cells and organs to xenobiotic stress, but unfortunately they can provide safety for cancer cells as well (MDR). These transporters are present in all pharmacological barriers in our body, where they determine pharmacokinetic and ADMET parameters of drugs and *in vitro* drug-transporter interactions can predict the tissue distribution of drug candidates. ABC transporters do not only cause problems when they function well, but mutations or the malfunction of the transporter proteins constitutes the basis of several diseases.

Chapter 1 describes these aspects in more detail and provides an overview of the methodology developed to measure the activity of ABC transporters in animal-, cellular- or vesicle systems, as well as a short introduction of the important recent discoveries concerning protein structure.

ABC transporters are at least partially located in lipid rafts, which are tightly packed domains in the plasma membrane enriched in sphingolipids and cholesterol. These lipid rafts can interact with the actin cytoskeleton, likely to confer stability or dynamic properties to lipid rafts; Moreover, several ABC transporters have been shown to link to actin themselves. **Chapter 2** provides an overview of the connections and interactions between ABC transporters, lipids, lipid rafts and actin.

Given that ABC transporters are located in lipid rafts, it is reasonable to hypothesize that lipids enriched in lipid rafts may support ABC transporter function. For example, it was previously shown that the activity of BCRP in Sf9 insect cell line is cholesterol dependent. **Chapter 3** concerns a study on cholesterol dependence of MRP1 performed in intact Neuro-2a and BHK-MRP1 cells as well as isolated membrane vesicles. We conclude that the activity of MRP1 is not affected by cholesterol since it was unaltered after both depletion and uploading of cholesterol.

In **Chapter 4** we investigated the sphingolipid dependence of MRP1 in BHK-MRP1 cells. It was shown before that MRP1 activity does not depend on the sphingolipid level of the membrane, as observed after short term sphingolipid depletion using myriocin. Interestingly, after long term depletion the activity of MRP1 did increase. Kinetic analysis of the transport process in isolated membrane vesicles revealed that it was not the substrate affinity that had changed (K_m value indicating the intrinsic activity of the protein), but the reaction speed had doubled (V_{max} indicating the number of active transporter molecules). This correlated well with the observation in whole cells that MRP1 was recruited to lipid rafts in long term myriocin treated cells.

The actin dependence of MRP1 and the closely related MRP2, MRP3 and MRP5 is the topic of **Chapter 5**. According to previous results the function of MRP1 in Neuro-2a as well as BHK-MRP1 cells is dependent on cortical actin. In the MDCKII cell line we could compare all four MRPs, since separate cell lines expressing one the four transporters were available. Surprisingly, MRP1 turned out to be independent of actin. This however was consistently explained by the observations that actin disruption did not affect lipid raft localization of MRP1 in MDCK cells, while it did in the other two cell lines. Also MRP3 and MRP5 were unaffected by actin disruption, but MRP2 lipid raft association decreased with a concomitant decrease of its activity. We speculate that MRP2 is directly linked to actin via radixin, as shown before in the context of *hyperbilirubinemia*, but not MDR.

The potential actin dependence study of ABC transporters was extended to Pgp in **Chapter 6** and studied in NIH 3T3 MDR G185 and SK-N-FI cell lines. The question was whether actin is important for Pgp function, as it is for MRP1 in certain cell lines and MRP2. Actin disruption did not affect Pgp efflux function or its localization in lipid rafts,

rendering Pgp is independent on actin. It should be noted that the extent of lipid raft association as well as the gradient fractions to which it mainly associates were different from those in the case of MRP1 in Neuro-2a cells.

Discussion

The aim of this thesis was to study efflux activity of ABC transporters as a function of their molecular environment and connections in the lateral (e.g. raft or non-raft localization) and transverse (cytoskeleton) plane of the membrane. The strength of this study is that it combines efflux activity and lipid rafts analysis as a function of modulation of lipid composition or the actin cytoskeleton, while many published studies focus either on function or localization of ABC transporters, especially in the case of actin modulation. Moreover, the effects of sphingolipids as important lipid raft components on ABC transporter function have received very limited attention thus far, in contrast to a possible role of cholesterol.

With our work we obtained supportive evidence for the model described in Chapter 2 (Fig. 2), which describes the various hypothetical pools of ABC transporters in connection to lipid rafts and the actin cytoskeleton. MRP1 in Neuro-2a and BHK-MRP1 cells behaved like it is in pool 3 (or 4). Our new results indicate that actin dependence is not specific for MRP1, because MRP1 in MDCKII cell line behaved like it is in pool 2 (Chapter 2, Fig. 2). In contrast to MRP1, MRP2 is actin dependent in this cell line and our results suggest that actin stress fibers are involved. However, the latter conclusion requires further evidence. The other pools described in this model, i.e. pools 1 and 5, also deserve attention. According to our results, all the tested ABC transporters are partially in pool 1 while their presence in pool 5 cannot be excluded in case of those ABC transporters that are actin dependent. We speculate that ABC transporters located outside the lipid rafts are not active, or have less activity than those inside lipid rafts. This is based on our consistent observations that a shift of a given ABC transporters out of an actin-stabilized lipid raft is always accompanied by a decrease of its activity (for example actin disruption and MRP2; Chapters 6). Also the opposite correlation was observed, i.e. an increase of MRP1

association with lipid rafts accompanied by an elevated efflux activity in the case of long term myriocin treatment (Chapter 4). The only case where there was no correlation was that of cholesterol-stabilized lipid rafts and MRP1 activity in Neuro-2a cells (Chapter 3).

It is interesting to speculate why ABC proteins would be more active when they are located in lipid rafts. It could be because they need some of the phospholipids which are enriched in lipid rafts or they need a more tightly packed lipid environment to suit their conformational requirements, potentially even involving dimer or multimer formation. If we extend this logic, we arrive at the point where we need to know more about the molecular structure of these proteins in different lipid environments. Then, it will be possible to fully understand the molecular mechanisms that control activity of ABC transporters. An interesting alternative explanation is that substrates, which are known to be hydrophobic, accumulate in lipid rafts during their entry into cells. If this would be the case, having ABC transporters in these domains would be beneficial in terms of their changes to encounter substrates.

Another positive aspect of this study which personally much appealed to me is the notion that our membrane vesicle studies and those in intact cells were mutually supportive, much like we anticipated and hoped it would be. Using the membrane vesicle approach we could analyze the kinetics of the MRP1 transport process (Chapter 4). This provided another clue for the notion that MRP1 in lipid rafts is more active than the non-raft associated protein. An increase of the V_{max} after long-term myriocin treatment indicates a larger pool of active MRP molecules, while in parallel experiments we indeed observed a recruitment of MRP1 to lipid rafts.

So what is actually important for the social life of ABC transporters? Connection to lipid rafts, specific phospholipids and actin appear to be players in the team of ABC proteins. As is the case for social relations among people – there are different kinds of people - it seems smart to believe that also different members of the ABC family need different connections. There is no unifying model. For example, MRP2 seems to prefer direct linkage to actin via ERM proteins, MRP1 lipid rafts which are stabilized by cortical actin while sphingolipids and cholesterol are dispensible, whereas BCRP is cholesterol activated and forms dimers, trimmers or tetramers. Moreover, not only are there differences

between ABC transporters, also for one specific ABC protein, its dependence on environmental factors may vary with the cell context, as became clear for MRP1.

Finally, potential application of our results are considered. I prefer to do science which sooner or later can be applied to the benefit of patients. Are these results useful? The answer is not straightforward. Clearly, nobody will consider using actin disrupting agents as drugs to cure patients with tumors in a hospital setting, or use cholesterol depleting agents for that matter. However, indirectly it is possible to use some of our results. As an example I mention Chapter 4, since this study can directly be applied to generate a standardized method for the preparation of membrane vesicles with higher signal to noise ratio, making the testing of drug-transporter interactions more effective. In Chapter 1, we have described why these *in vitro* test systems are so important in drug development.

In conclusion, our research has led to a better understanding of the molecular parameters that belong to the social life of ABC transporters and how they appear to influence their activity in a variety of ways and depending on the cellular background. Moreover, this research may lead to improved tools for drug testing in the future.

Nederlandse samenvatting

Kanker is een van de meest ernstige ziektebeelden in de ontwikkelde landen and de meeste voor de hand liggende behandeling is chemotherapie, vooral in geval van metastases. Als niet alle tumorcellen worden gedood door de behandeling, kan de tumor terugkeren en dan zelfs als een resistente tumor. Dat levert een groot probleem op voor de vervolg behandeling. Resistentie van tumorcellen kan worden veroorzaakt door ATP-bindings cassette (ABC) transporters. Deze bevindingen vestigen al lange tijd de aandacht van academische onderzoekers en farmaceutische bedrijven op deze problematiek en onderzoek naar oplossingen. Vandaag de dag weten we dat ABC transporters membraan eiwitten zijn met meerdere trans-membraan fragmenten en dat deze eiwitten de capaciteit hebben om moleculen met zeer variabele structuren uit de cel te pompen. ABC transporters vormen één van de grootste eiwit families en zijn aanwezig in alle bekende organismen van bacteriën tot de mens. De aanwezigheid van deze transporters is een van de belangrijkste verdediging mechanismen om cellen te beschermen tegen stress door lichaamsvreemde moleculen, maar maakt helaas ook verdediging van kanker cellen tegen chemotherapie mogelijk (‘MDR’). Deze transporters zijn aanwezig in alle farmacologische barrières in ons lichaam, waar ze farmacokinetische en ‘ADMET’ eigenschappen van geneesmiddelen bepalen. *In vitro* geneesmiddel-transporter interacties kunnen orgaan en weefsel verdeling van kandidaat geneesmiddelen voorspellen. ABC transporters veroorzaken niet allen problemen als ze goed werken, maar ook mutaties en slecht functionerende transporter eiwitten vormen de basis van diverse ziektes. Hoofdstuk 1 behandelt deze aspecten in detail en geeft een overzicht van de methodologie die is ontwikkeld om de activiteit van ABC transporters te meten in dier-, cel- en membraan-modellen, en tevens geeft dit hoofdstuk een kort overzicht van de belangrijkste recente ontwikkelingen op het gebied van ABC eiwit structuur.

ABC transporters zijn gedeeltelijk gelokaliseerd in zogenaamde lipiden rafts, membraan domeinen die worden gekenmerkt door een dichte pakking van lipiden en verrijkt aan sfingolipiden en cholesterol. Deze lipiden rafts kunnen een interactie aangaan met het actine cytoskelet, en hierdoor worden lipiden rafts gestabiliseerd of krijgen ze juist dynamische eigenschappen; Daar komt bij dat van sommige ABC transporters bekend is

dat ze zelf een direct link met actine hebben. Hoofdstuk 2 geeft een overzicht van de connecties en interacties tussen ABC transporters, lipiden, lipiden rafts en actine.

Aangezien ABC transporters in lipiden rafts voorkomen, is het aannemelijk dat de lipiden die verrijkt zijn in lipiden rafts mogelijkerwijs nodig zijn voor de functie van ABC transporters. In dat opzicht is al aangetoond dat de activiteit van BCRP in Sf9 insecten cellen cholesterol afhankelijk is. In Hoofdstuk 3 wordt de cholesterol afhankelijkheid van MRP1 in Neuro-2a en BHK-MRP1 cellen en tevens in membraan vesicles geïsoleerd uit BHK-MRP1 cellen onderzocht. We komen tot de conclusie dat MRP1 niet afhankelijk is van cholesterol aangezien zowel depletie als verhoging van cholesterol in de cellen geen verandering van activiteit laat zien.

In Hoofdstuk 4 onderzoeken we de sfingolipiden afhankelijkheid van MRP1 in BHK-MRP1 cellen. Eerder was aangetoond dat MRP1 niet afhankelijk is van het sfingolipiden niveau in de membraan, zoals werd gevonden bij korte termijn sfingolipiden depletie met myriocin. Interessant genoeg blijkt MRP1 activiteit wel toe te nemen na lange termijn sfingolipiden depletie. Kinetische analyse van het transport proces in geisoleerde membraan vesicles liet zien dat niet de affiniteit voor substraat was veranderd (Km waarde die de intrinsieke activiteit van het eiwit aangeeft), maar dat de reactie snelheid was verdubbeld (Vmax waarde die het aantal actieve transporter moleculen aangeeft). Deze waarneming is goed te rijmen met het gegeven dat in hele cellen MRP1 werd gerekruteerd in lipiden rafts in lange termijn myriocin behandelde cellen.

De actine afhankelijkheid van MRP1 en de gerelateerde transporters MRP2, MRP3 en MRP5 staat centraal in Hoofdstuk 5. Volgens eerder behaalde resultaten is de functie van MRP1 in Neuro-2a en BHK-MRP1 cellen afhankelijk van corticaal actine. In MDCKII cellen kunnen we alle 4 MRP's vergelijken, omdat 4 cellijnen met elk over expressie van één van de MRP's beschikbaar zijn. Geheel onverwacht bleek MRP1 onafhankelijk van actine in deze cellijn. Dit was op zich heel consistent met het resultaat dat actine afbraak de lokalisatie van MRP1 in lipiden rafts niet beïnvloedde in deze cellen, in tegenstelling tot de situatie in Neuro-2a en BHK-MRP1 cellen. Ook MRP3 en MRP5 werden niet door actine afbraak in functie gehinderd, maar MRP2 functie verminderde en dat ging gepaard met een afname van de lipiden raft associatie van MRP2. We speculeren dat MRP2 direct aan het

actine cytoskelet is verbonden via het molecuul radixin, wat eerder is aangetoond in de context van hyperbilirubinemie.

Een mogelijk actine afhankelijkheid van ABC transporters wordt verder uitgebreid naar Pgp in Hoofdstuk 6 en daarin onderzocht in NIH 3T3 en SK-N-FI cellen. De vraag was of actine van belang is voor Pgp functie, zoals voor MRP1 in sommige cellen en MRP2. Actine afbraak veranderde Pgp gemedieerde efflux niet en had ook geen effect op de localisatie van Pgp in lipiden rafts. Pgp is dus onafhankelijk van actine. Hierbij kan worden opgemerkt dat zowel de mate van lipiden raft associatie van Pgp in controle cellen als de gradiënt fracties waarin Pgp voornamelijk voorkomt verschillen van die in het geval van MRP1 in Neuro-2a cellen.

Discussie

Het doel van dit proefschrift was het bestuderen van de efflux activiteit van ABC transporters als functie van hun moleculaire omgeving en connecties in het laterale vlak (bijvoorbeeld lipiden raft associatie) en het transversale vlak (cytoskelet) van de membraan. De kracht van dit onderzoek ligt in de combinatie van efflux activiteit en lipiden raft analyse als functie van modulatie van lipiden compositie en het cytoskelet, waar veel gepubliceerde studies zich beperken tot ofwel functie ofwel lokalisatie van ABC transporters, vooral in het geval van actine modulatie. Daar komt bij dat de effecten van sfingolipiden als belangrijke componenten van lipiden rafts op ABC transporter functie tot nu toe weinig aandacht hebben gekregen in de literatuur, in tegenstelling tot effecten van cholesterol.

Met dit onderzoek werd ondersteunend bewijs verkregen voor het model dat werd geponeerd in Hoofdstuk 2 (Figuur 2), dat de verschillende hypothetische pools van ABC transporters in samenhang met lipiden rafts en het actine cytoskelet beschrijft. MRP1 in Neuro-2a en BHK-MRP1 cellen gedraagt zich alsof het zich in pool 3 (of 4) bevindt. Onze nieuwe resultaten laten zien dat actine afhankelijkheid niet universeel is voor MRP1, aangezien MRP1 in MDCKII cellen zich gedraagt alsof het zich in pool 2 bevindt. In tegenstelling tot MRP1, is MRP2 actine afhankelijk in deze cellen en onze resultaten

suggereren dat actine stress fibers hierbij betrokken zijn. Maar deze conclusie kan pas getrokken worden na verder diepgaand onderzoek. De overige pools beschreven in het model, dus pool 1 en 5, vragen ook de aandacht. Uit onze resultaten volgt dat alle ABC transporters die we hebben onderzocht zich gedeeltelijk in pool 1 bevinden, terwijl hun voorkomen in pool 5 niet kan worden uitgesloten, voor zover het de ABC transporters betreft die actine afhankelijk bleken te zijn. We speculeren dat ABC transporters die zich buiten de lipiden rafts bevinden niet of minder actief zijn dan die in de lipiden rafts. Deze speculatie is gebaseerd op de gegevens dat een shift van een ABC transporter uit actine gestabiliseerde lipiden rafts altijd gepaard gaat met verlies van functie (bijvoorbeeld actine afbraak en MRP2; Hoofdstuk 6). Het tegenovergestelde bleek ook op te gaan, dat wil zeggen dat ABC transporter functie toenam in het geval van rekrutering van deze transporter in lipiden rafts in het geval van lange termijn myriocin behandeling (Hoofdstuk 4). Het enige geval waarin geen correlatie werd gevonden was tussen cholesterol-gestabiliseerde lipiden rafts en MRP1 functie (Hoofdstuk 3). Het is van belang om te speculeren waarom ABC eiwitten meer actief zouden zijn als ze zich in lipiden rafts bevinden. Het zou te maken kunnen hebben met een behoefte aan bepaalde fosfolipiden die zich in lipiden rafts bevinden of met een dichte pakking van lipiden die nodig kan zijn om de ABC transporter in de juiste conformatie te brengen/houden, of zelfs voor dimeer of multimeer vorming. Als we deze logische redenering voortzetten, komen we bij het punt uit waarop we moeten constateren dat we meer moeten weten over de moleculaire structuur van deze eiwitten in verschillende lipiden omgevingen. Dan zal het mogelijk worden om de moleculaire mechanismen die de activiteit van ABC transporters sturen precies te leren kennen en begrijpen. Een interessante alternatieve verklaring voor het voorkomen van ABC transporters in lipiden rafts is overigens dat substraten, die immers hydrofoob zijn, accumuleren in lipiden rafts. Als dat zo is, dan is het logisch dat ABC transporters op hun plaats zijn in deze lipiden rafts om zo hun substraten te kunnen tegenkomen.

Een ander pluspunt van dit onderzoek dat mij persoonlijk sterk aanspreekt is het gegeven dat onze membraan vesicle studies goed aansluiten bij het onderzoek in intacte cellen en dat de resultaten uit beide typen onderzoek elkaar ondersteunen, zoals we van te voren hadden gehoopt. Met de membraan vesicle benadering werd het mogelijk om de

kinetiek van het transport proces gemedieerd door MRP1 te bepalen (Hoofdstuk 4). Die analyse bracht een nieuw stuk bewijs voor de hypothese dat MRP1 in lipiden rafts actiever is dan daarbuiten. Een toename van de Vmax na lange termijn myriocin behandeling kan worden geïnterpreteerd als een grotere pool van actieve MRP1 moleculen, terwijl we weten dat de totale plasma membraan pool gelijk bleef en we in parallelle experimenten in intacte cellen inderdaad een rekrutering van MRP1 in lipiden rafts zagen. Wat is er nu belangrijk voor het sociale leven van een ABC transporter? Verbindingen met het actine cytoskelet, lipiden rafts en specifieke fofsolipiden blijken belangrijke spelers te zijn in het team van ABC eiwitten. Net zoals bij het sociale leven van mensen – er zijn wat dat betreft grote verschillen in behoefte tussen mensen – blijkt het verstandig om er van uit te gaan dat ook verschillende leden van de ABC transporter familie verschillende behoeften hebben met betrekking tot interacties met de omgeving. Er is dus geen unificatie theorie. MRP2 bijvoorbeeld prefereert een directe link naar actine (stress fibers?) via ERM eiwitten, MRP1 prefereert lipiden rafts die worden gestabiliseerd door corticaal actin terwijl sfingolipiden en cholesterol kunnen worden gemist. BCRP daarentegen wordt gestimuleerd door cholesterol. Daar komt nog bij dat er niet alleen verschillen zijn tussen ABC transporters onderling, maar voor een bepaalde ABC transporter geldt bovendien dat de afhankelijkheid van bepaalde omgevingsfactoren kan verschillen in de context van het celtype, zoals bleek voor MRP1.

Tot slot willen we de potentiële toepassingen van ons onderzoek bespreken. Ik geef er de voorkeur aan om het soort wetenschap te bedrijven dat vroeger of later kan worden toegepast voor het welzijn van patiënten. Zijn onze resultaten dan zinvol? Het antwoord op deze vraag is niet eenvoudig. Het mag duidelijk zijn dat niemand zal overwegen om actine afbrekende middelen in te zetten als geneesmiddelen om patiënten met tumoren te behandelen in het ziekenhuis. Of om cholesterol depletie toe te passen in deze context. Maar op een indirecte wijze is het wel degelijk mogelijk om onze resultaten toe te passen. Als voorbeeld wil ik Hoofdstuk 4 noemen, omdat deze studie direct kan worden toegepast om een gestandaardiseerde methode te ontwikkelen voor het maken van membraan vesicles met een hogere signaal-ruis verhouding die het testen van geneesmiddel-transporter interacties effectiever zullen maken. In Hoofdstuk 1 hebben we aangegeven waarom deze

in vitro systemen zo belangrijk zijn voor geneesmiddel ontwikkeling. Samenvattend kan worden gezegd dat ons onderzoek heeft bijgedragen aan een beter begrip van de moleculaire parameters die kunnen worden gerekend tot het sociale leven van de ABC transporter en hoe deze factoren de activiteit van ABC eiwitten op verschillende wijze beïnvloeden, mede afhankelijk van de cel context. Daar komt bij dat ons onderzoek kan leiden tot verbeterde tools voor het testen van geneesmiddelen.

Magyar nyelvű összefoglaló

Mik azok az ABC transzporterek? Talán legegyszerűbben Glavinas Hristos, egykori munkatársam fogalmazta meg: Képzeljünk el egy pohár vizet! Ha a vízbe cseppentünk egy csepp tintát és sokáig várunk, a tinta egyenletesen el fog oszlani a vízben. Ha a tintacseppet, vagy egy tabletta gyógyszert valaki bevesz és várunk, azt fogjuk tapasztalni, hogy nem oszlik el egyenletesen! Vannak szövetek ahol a gyógyszer jelen lesz és vannak, ahol nem. Miért van ez így? A válasz a minden sejtünk falában megtalálható ABC transzporterekben (vagyis ATP kötő „kazetta") rejlik. Ezek olyan aktív transzporterek, amelyek ATP hidrolízisből nyert energia segítségével képesek anyagokat átjuttatni a membrán egyik oldaláról a másikra koncentrációgradiens ellenében. Egyszerűbben fogalmazva kipumpálják a sejtből a mérgeket, különböző molekulákat, gyógyszereket. Ezért nem oszlanak el a gyógyszerek egyenletesen.

Számos, az ABC transzporterek hiánya miatt kialakuló betegség megértése vezetett el bennünket annak felismeréséig, mennyire fontosak ezek a fehérjék. Legismertebb ezek közül a tumorok ún. multidrog rezisztenciája. Ebben az esetben a tumorsejtek az egészséges sejtekhez képest túltermelnek egy vagy több ABC transzporter fehérjét, ezáltal képesek a sejtekből kipumpálni például a kemoterápiás gyógyszermolekulákat, megakadályozva a hatóanyag felhalmozódását, ami végül el tudná pusztítani a rákos sejtet. Így megvédik magukat a kemoterápiával szemben és rezisztensé válnak. Ugyanakkor a tumorsejtek ezen tulajdonsága segít demonstrálni, miért olyan általánosan elterjedtek ezek a fehérjék. Ugyanis egy pumpaként működő membránfehérje, ami eltávolítja a sejtekből a mérgező vegyületeket, védelmet ad a környezettel szemben (lsd. xenobiotikumokkal szembeni védelem). Ennek köszönhetően a mai gyógyszer hatóanyag fejlesztések elengedhetetlen részét jelentik a drog-transzporter kölcsönhatás vizsgálatok *in vitro* és *in vivo* módszerekkel.

Az ABC transzporterek az egyik legnagyobb családot alkotják a fehérjék között. Az emberi genomban 49 ABC fehérjét kódoló gént találtak, de éppúgy megtalálhatóak minden ismert organizmusban a mikróbáktól az emberig. Mivel ilyen fontosak és jelentősen befolyásolják a farmakológiailag aktív vegyületek felszívódását (abszorpció), eloszlását

(disztribúció), metabolizmusát és szervezeten belüli kiválasztását (exkréció) (ADME), nagy szükség van a működésük megismerésére. Az elmúlt 10-15 évben sok kutatócsoport foglalkozott ezzel a témával. Van, amelyik arra specializálódott, hogy pontos szerkezeti képet adjon róluk, mások pedig a különböző gyógyszerjelöltek és transzporterek közti kölcsönhatásokat mérték.

Csoportunkkal a négy éves munka során azt vizsgáltuk Groningenben, hogy a sejtmembrán és a sejt "csontvázának" egyes alkotóelemei hogyan tudják befolyásolni néhány ilyen fehérje működését. A doktori disszertáció célja az ABC transzporterek aktivitásának membránbéli molekuláris környezettől való függésének vizsgálata. Milyen kapcsolataik vannak és melyek fontosak a működésükhöz. Ezen fehérjék pontosabb ismerete segíthet legyőzni a multidrog rezisztenciát, ami nagyon fontos lépés a sikeres kemoterápia és a rák legyőzése felé vezető úton. Először röviden összefoglalom mely fehérjékkel foglalkoztunk, majd ezután az egyes fejezetek tartalma kerül bemutatásra.

Az ABC transzporterek közül a P-glycoprotein (Pgp, ABCB1, MDR1) fehérjét írták le elsőként 1976-ban. Megfigyelték, hogy multidrog rezisztenciát okoz rákos sejtekben. A glikozilált P-gp fehérjék a polarizált sejtek plazmamembránjában helyezkednek el, olyan élettanilag kulcsfontosságú helyeken, mint az epecsatornákban, a bélbolyhokban, a vese proximális tubulusok hámsejtjeiben és a vér-agy-gát endotél sejtjeiben. Ez a fehérje fontos szerepet tölt be a sejtek, szövetek védelmében a mérgező toxikus molekulákkal szemben.

A Multidrug Resistance-associated Protein 1 (MRP1, ABCC1) fehérjét a multidrog rezisztencia egyik alapfehérjéjeként ismerték meg. Hasonlóan az Pgp-hez, az MRP1-et expresszáló tumorok is ellenállóak a kezelésekkel (főleg hidrofób gyógyszerekkel) szemben. Jellemző szubsztrátjai még a szerves anionok: glutation, glukoronát vagy szulfát konjugátumok. Számos külső és belső mérgező anyag válik semlegessé a fent említett vegyületekkel kapcsolódva, amelyeket aztán az MRP2 (ABCC2, cMOAT) választ ki az epébe és a vizeletbe. Az MRP2 hibái *hyperbilirubinémiát* vagy *Dubin-Johnson szindrómá*t okozhatnak. Ez a transzporter is befolyásolja a daganatellenes gyógyszerek hatását. Az MRP3 (ABCC3) és MRP5 (ABCC5) szintén szerves anionokat, glutation konjugátumokat transzportál, szubsztrátjai főleg a glukoronát konjugátumok.

Az **első fejezet**ben az ABC fehérjék családjának részletesebb leírása mellett bemutatom, hogy üzletileg mire használhatóak. Néhány biotech cég fő profilja az ezekkel a fehérjékkel készült in vitro tesztrendszerek gyártása és használata, hasonlóképp a gyógyszeriparban is nélkülözhetetlenek. Mivel ezt olyan szervezetek, mint az FDA is erősen támogatja, nagyon fontos megérteni az ABC transzporterek működését. Azt is kifejtem, hogyan lehet ezen fehérjék specifikus aktivitását tanulmányozni állati, sejt alapú és vezikuláris rendszerekben, majd eljutok az atomi szintű szerkezetük megértésének fontosságáig.

Bár az első fejezetben rövid ismertetőt adok a sejt aktin „csontvázáról", témabeli fontosságáról és kitérek az úgynevezett lipid raftokra is, ezek részletes áttekintése a **második fejezet**ben található.

A **harmadik fejezet**ben az MRP1 aktivitásának koleszterin függését tanulmányozzuk. Ezek a fehérjék legalább részben lipid raftokban helyezkednek el, amik koleszterinben és szfingolipidekben gazdag membrán egységek. Lehetséges-e, hogy ezen lipidek szükségesek a transzporterek működéséhez és ha igen, hogyan? Korábbi eredmények szerint a BCRP aktivitása rovar sejtvonalban koleszterin függő, ezt most sejt és vezikula rendszereken vizsgáljuk az MRP1 esetében emlős (bébi hörcsög vese, BHK) sejtvonalban. Mint kiderült, az MRP1 aktivitása változatlan marad mind a sejtmembrán koleszterin tartalmának csökkentése, mind pedig növelése esetén.

Egy rövid kitérővel szeretném megmagyarázni miért dolgoztunk többféle sejtvonallal. 2008-ban a zölden fluoreszkáló, medúza fehérje (GFP) klónozásáért adta át a Nobel-díjat XVI. Károly Gusztáv svéd király. Az eredeti munkát, miszerint a medúza GFP fehérje génjét bevitték más élőlények sejteibe, manapság már könnyű elvégezni, magam is csináltam másodéves egyetemista koromban. A cukorbetegség kezelésére használt humán inzulint is ilyen transzgenikus baktériumok termelik. Hasonlóképp a humán ABC transzporterek is „termeltethetőek" más fajok immortalizált sejtvonalaiban, így model sejtvonalak hozhatóak létre különböző sejttípusok tulajdonságaival, amelyek segítségével kiválóan lehet vizsgálni, modellezni ezen fehérjék környezetét és működését.

A **negyedik fejezet**ben továbbra is az MRP1 fehérje aktivitását kutatjuk, de más lipidek, a szfingolipidek mennyiségének függvényében. A transzport aktivitás ezek szintjétől független, ugyanakkor ellentétben a rövid távú szfingolipid csökkentéssel, hosszú

távon erősödik az MRP1 aktivitása mind a sejtekben, mind pedig a vezikulákban. A transzport kinetikai elemzése kiderítette, hogy valójában nem a szubsztrát affinitás („egy darab fehérje aktivitása") változik, hanem a reakció sebessége duplázódik (nőtt az aktív fehérjék száma). Ez az eredmény a BCRP-koleszterin munkához hasonlóan hasznosítható, hiszen segítségével jobb jel-zaj arányú membrán preparátumokat lehet készíteni, ez mégsem jutott el a szabadalmi oltalomig. Ennek oka főleg a szabadalom védhetőségében rejlik.

Az MRP1 és „testvérei", a hozzá közel álló MRP2, MRP3 és MRP5 aktin érzékenységét vizsgáljuk az **ötödik fejezet**ben. Korábbi eredményeink szerint a humán MRP1 érzékeny a sejtmembrán alatti aktin hálózat sértetlenségére BHK sejtvonalban. Kutya vese sejtvonalban (MDCKII), ahol mind a négy fehérje tulajdonságait összehasonlíthatjuk, viszont nem. Ez egyrészt meglepő, másrészt következetes eredmény, mert BHK sejtek esetén csökken az MRP1 lipid raftokban való elhelyezkedése az aktin kapcsolat megszűnése után, itt viszont ez az ok nincs jelen. Ebben a rendszerben csak az MRP2 membránbéli helye változik, így aktivitása csökken. Az MRP2, mint kiderült, valószínűleg az egész sejtet átívelő aktin formához kapcsolódik egy radixin nevű fehérje segítségével, ami korábban kapcsolatban állt a *hiperbilirubinémiával* is, de a multidrog rezisztenciával összefüggésben még nem írták le. Ez a munka azt is bebizonyítja, hogy az MRP1 aktin érzékenysége sejtfüggő.

Ezt a kutatást terjesztjük ki a **hatodik fejezet**ben a Pgp esetére is egy egér és egy humán sejtvonalban (NIH 3T3, SK-N-FI). Ennek tanulmányozása során már nem csak főleg áramlási citometria és a fluoreszcencia aktivált sejt szeparálást, valamint konfokális lézer pásztázó mikroszkópot, hanem elektronmikroszkópot is használunk a sejtmembrán morfológiai változásainak pontosabb megsimeréséhez.

Továbbá mind a harmadik, negyedik és ötödik fejezet is egyre több és több bizonyítékot szolgáltat a második fejezetben kifejtett modellünkhöz (Fig. 2.). Eszerint azok az ABC transzporterek, melyek nem lipid raftokban helyezkednek el, nem- vagy kevésbé aktívak. Ennek pontos oka még felderítésre vár, lehet hogy a lipid raftok tömörebb szerkezete vagy az ott megtalálható speciális lipidek kölcsönhatása szükséges a transzporterek megfelelő konformációjának kialakításához. Végső soron ezekre a

kérdésekre a fehérjék atomi szintű szerkezeti ismerete adhat választ, amely gondolattal visszakanyarodhatunk Szent-Györgyi Albert 1960-ban (!) megfogalmazott mondatához (10. Stelling).

Munkám nagy része tehát abból állt, hogy ezeket a fejezeteket (projekteket vagy cikkeket) csapatban dolgozva megalkossam, legyőzzem a felmerülő tudományos és technikai problémákat, létrehozzam a szükséges mérési módszereket és tesztrendszereket, és végül úgynevezett peer-reviewed (nemzetközileg lektorált, szakmailag elismert, jó impakt faktorú) folyóiratokban elfogadtassam.

Végül néhány mondatban megemlíteném az eredmények alkalmazhatóságát, hiszen én nem csak publikálni, hanem alkalmazni is szeretem a tudományt, továbbá a hosszú távú cél a betegek gyógyítása. Hasznosak ezek az eredmények? A válasz nem egyszerű. Közvetlenül nem hasznosak, természetesen senki sem szeretné a kórházban a betegek tumorjait aktin vagy koleszterin moduláló szerekkel kezelni... Közvetve viszont használhatóak, példaként említeném a negyedik fejezetet. Ezzel az eredménnyel új, jobb jel-zaj arányú membrán preparátumokat lehet létrehozni, amivel hatékonybban mérhetőek a gyógyszer-transzporter kölcsönhatások. Az első fejezetben kifejtettem miért fontosak ezek a gyógyszeriparban. Továbbá fontos állomást jelenthetnek ezen fehérjék működésének modellezéshez vezető úton. A jövőben hasznos lenne például az MRP1 szerkezetének meghatározása negyedik fejezetben leírt körülmények mellett.

A kutatásaink segítettek megérteni az ABC transzporterek molekuláris kölcsönhatásait, kapcsolatait, „társadalmi életüket”, hogy ezek hogyan tudják az aktivitásukat befolyásolni és milyen módokon, milyen sejtrendszerben. Emellett ezek az eredmények fejlettebb gyógyszer tesztelő módszerekhez vezethetnek.

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About the author

Péter Mészáros was born in Székesfehérvár, in Hungary on 25 July, 1983. He studied biophysics at the University of Szeged, got in contact with biotechnology during this time and worked nearly 3 years at Solvo Biotechnology. In this position he studied the biochemistry of ABC transporters and measured drug-transporter interactions. Finally, at the end of 2007 he joined the group of Jan Willem Kok as a PhD student at the University Medical Center Groningen in the Netherlands. In this four years he did research and development in the field of ABC transporters in the context of their lipid and protein interactions beside keeping his interest in business and stock market.

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